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(75) Inventors/Applicants *(for US only)*: ARMSTRONG, Marcy, E. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). KEYS, Robert, D. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). LEWIS, John, A. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). LIU, Margaret, A. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). McCLEMENTS, William, L. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).

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~~(54) Title:~~ A POLYNUCLEOTIDE HERPES VIRUS VACCINE

(57) Abstract

Genes encoding herpes simplex virus type 2 (HSV-2) proteins were cloned into eukaryotic expression vectors to express the encoded proteins in mammalian muscle cells *in vivo*. Animals were immunized by injection of these DNA constructs, termed polynucleotide vaccines or PNV, into their muscles. In a DNA titration, it was found that a single immunization of ≥ 0.5 μg of (one) PNV, gave > 90 % seroconversion by ten weeks post immunization. Immune antisera neutralized both HSV-2 and HSV-1 in cell culture. When animals were challenged with HSV-2, significant ($p < .001$) protection from lethal infection was achieved following PNV vaccination.

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TITLE OF THE INVENTION

A POLYNUCLEOTIDE HERPES VIRUS VACCINE

BACKGROUND OF THE INVENTION

A major obstacle to the development of vaccines against viruses, particularly those with multiple serotypes or a high rate of mutation, against which elicitation of neutralizing and protective immune responses is desirable, is the diversity of the viral external proteins among different viral isolates or strains. Since cytotoxic T-lymphocytes (CTLs) in both mice and humans are capable of recognizing epitopes derived from conserved internal viral proteins [J.W. Yewdell *et al.*, *Proc. Natl. Acad. Sci. (USA)* **82**, 1785 (1985); A.R.M. Townsend, *et al.*, *Cell* **44**, 959 (1986); A.J. McMichael *et al.*, *J. Gen. Virol.* **67**, 719 (1986); J. Bastin *et al.*, *J. Exp. Med.* **165**, 1508 (1987); A.R.M. Townsend and H. Bodmer, *Annu. Rev. Immunol.* **7**, 601 (1989)], and are thought to be important in the immune response against viruses [Y.-L. Lin and B.A. Askonas, *J. Exp. Med.* **154**, 225 (1981); I. Gardner *et al.*, *Eur. J. Immunol.* **4**, 68 (1974); K.L. Yap and G.L. Ada, *Nature* **273**, 238 (1978); A.J. McMichael *et al.*, *New Engl. J. Med.* **309**, 13 (1983); P.M. Taylor and B.A. Askonas, *Immunol.* **58**, 417 (1986)], efforts have been directed towards the development of CTL vaccines capable of providing heterologous protection against different viral strains.

It is known that CTLs kill virally-infected cells when their T cell receptors recognize viral peptides associated with MHC class I and or class II molecules. These peptides can be derived from endogenously synthesized viral proteins, regardless of the protein's location or function within the virus. By recognition of epitopes from conserved viral proteins, CTLs may provide heterologous protection.

Many infectious disease causing agents can, by themselves, elicit protective antibodies which can bind to and kill, render harmless, or cause to be killed or rendered harmless, the disease causing agent and its byproducts. Recuperation from these diseases usually results in long-lasting immunity by virtue of protective antibodies generated against the highly antigenic components of the infectious agent.

Protective antibodies are part of the natural defense mechanism of humans and many other animals, and are found in the blood as well as in other tissues and bodily fluids. It is the primary function of most vaccines to elicit protective antibodies against infectious agents and/or their byproducts, without causing disease.

Most efforts to generate CTL responses have either used replicating vectors to produce the protein antigen within the cell [J.R. Bennink *et al., ibid.* **311**, 578 (1984); J.R. Bennink and J.W. Yewdell, *Curr. Top. Microbiol. Immunol.* **163**, 153 (1990); C.K. Stover *et al., Nature* **351**, 456 (1991); A. Aldovini and R.A. Young, *Nature* **351**, 479 (1991); R. Schafer *et al., J. Immunol.* **149**, 53 (1992); C.S. Hahn *et al., Proc. Natl. Acad. Sci. (USA)* **89**, 2679 (1992)], or they have focused upon the introduction of peptides into the cytosol [F.R. Carbone and M.J. Bevan, *J. Exp. Med.* **169**, 603 (1989); K. Deres *et al., Nature* **342**, 561 (1989); H. Takahashi *et al., ibid.* **344**, 873 (1990); D.S. Collins *et al., J. Immunol.* **148**, 3336 (1992); M.J. Newman *et al., ibid.* **148**, 2357 (1992)]. Both of these approaches have limitations that may reduce their utility as vaccines. Retroviral vectors have restrictions on the size and structure of polypeptides that can be expressed as fusion proteins while maintaining the ability of the recombinant virus to replicate [A.D. Miller, *Curr. Top. Microbiol. Immunol.* **158**, 1 (1992)], and the effectiveness of vectors such as vaccinia for subsequent immunizations may be compromised by immune responses against vaccinia [E.L. Cooney *et al., Lancet* **337**, 567 (1991)]. Also, viral vectors and modified pathogens have inherent risks that may hinder their use in humans [R.R. Redfield *et al., New Engl. J. Med.* **316**, 673 (1987); L. Mascola *et al., Arch. Intern. Med.* **149**, 1569 (1989)]. Furthermore, the selection of peptide epitopes to be presented is dependent upon the structure of an individual's MHC antigens and, therefore, peptide vaccines may have limited effectiveness due to the diversity of MHC haplotypes in outbred populations.

Benvenisty, N., and Reshef, L. [*PNAS* **83**, 9551-9555, (1986)] showed that $CaCl_2$ precipitated DNA introduced into mice intraperitoneally (i.p.), intravenously (i.v.) or intramuscularly (i.m.) could be expressed. The intramuscular (i.m.) injection of DNA expression

vectors in mice has been demonstrated to result in the uptake of DNA by the muscle cells and expression of the protein encoded by the DNA [J.A. Wolff *et al.*, *Science* **247**, 1465 (1990); G. Ascadi *et al.*, *Nature* **352**, 815 (1991)]. The plasmids were shown to be maintained episomally and did not replicate. Subsequently, persistent expression has been observed after i.m. injection in skeletal muscle of rats, fish and primates, and cardiac muscle of rats [H. Lin *et al.*, *Circulation* **82**, 2217 (1990); R.N. Kitsis *et al.*, *Proc. Natl. Acad. Sci. (USA)* **88**, 4138 (1991); E. Hansen *et al.*, *FEBS Lett.* **290**, 73 (1991); S. Jiao *et al.*, *Hum. Gene Therapy* **3**, 21 (1992); J.A. Wolff *et al.*, *Human Mol. Genet.* **1**, 363 (1992)]. The technique of using nucleic acids as therapeutic agents was reported in WO90/11092 (4 October 1990), in which naked polynucleotides were used to vaccinate vertebrates.

Recently, the coordinate roles of B7 and the major histocompatibility complex (MHC) presentation of epitopes on the surface of antigen presenting cells in activating CTLs for the elimination of tumors was reviewed [Edgington, *Biotechnology* **11**, 1117-1119, 1993]. Once the MHC molecule on the surface of an antigen presenting cell (APC) presents an epitope to a T-cell receptor (TCR), B7 expressed on the surface of the same APC acts as a second signal by binding to CTLA-4 or CD28. The result is rapid division of CD4+ helper T-cells which signal CD8+ T-cells to proliferate and kill the APC.

It is not necessary for the success of the method that immunization be intramuscular. Thus, Tang *et al.*, [*Nature*, **356**, 152-154 (1992)] disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice. Furth *et al.*, [*Analytical Biochemistry*, **205**, 365-368, (1992)] showed that a jet injector could be used to transfect skin, muscle, fat, and mammary tissues of living animals. Various methods for introducing nucleic acids was recently reviewed [Friedman, T., *Science*, **244**, 1275-1281 (1989)]. See also Robinson *et al.*, [*Abstracts of Papers Presented at the 1992 meeting on Modern Approaches to New Vaccines, Including Prevention of AIDS*, Cold Spring Harbor, p92], where the im, ip, and iv administration of

avian influenza DNA into chickens was alleged to have provided protection against lethal challenge. Intravenous injection of a DNA:cationic liposome complex in mice was shown by Zhu *et al.*, [*Science* **261**, 209-211 (9 July 1993); see also WO93/24640, 9 Dec. 1993] to result in systemic expression of a cloned transgene. Recently, Ulmer *et al.*, [*Science* **259**, 1745-1749, (1993)] reported on the heterologous protection against influenza virus infection by injection of DNA encoding influenza virus proteins.

Wang *et al.*, [*P.N.A.S. USA* **90**, 4156-4160 (May, 1993)] reported on elicitation of immune responses in mice against HIV by intramuscular inoculation with a cloned, genomic (unspliced) HIV gene. However, the level of immune responses achieved was very low, and the system utilized portions of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter and portions of the simian virus 40 (SV40) promoter and terminator. SV40 is known to transform cells, possibly through integration into host cellular DNA. Thus, the system described by Wang *et al.*, is wholly inappropriate for administration to humans, which is one of the objects of the instant invention.

WO 93/17706 describes a method for vaccinating an animal against a virus, wherein carrier particles were coated with a gene construct and the coated particles are accelerated into cells of an animal.

Recent efforts to develop subunit vaccines for herpes simplex virus (HSV) have focused on novel expression and presentation of viral antigens; especially the viral glycoproteins. **[for review see** Burke, R.L., 1993, Sem.In Virol., 4, pp.187-197] Recombinant HSV glycoproteins expressed by a variety of systems including yeast (Kino., Y.C. et al., 1989, Vaccine, 7, pp.155-160), insect cells (Ghiasi, H. et al., 1991, Arch.Virol., 121, pp.163-178), and mammalian cells (Burke, R.L., 1991, Rev.Infect.Dis., 13, S906-S911; Lasky, L.A., 1990, J.Med.Virol., 31, pp.59-61) have been shown to elicit protective immunity in animal models. Clinical trials of a recombinant HSV-2 glycoprotein D (gD) produced in Chinese hamster ovary cells have shown that the vaccine induces an antibody response in naive individuals and stimulates the pre-

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existing response in both HSV-1 and HSV-2 seropositive individuals. (Straus, S.E.et al., 1993, J.Infect.Dis., 167, pp.1045-1052)

An alternate approach to subunit vaccination has been the use of live virus vectors for delivery of HSV antigens. Vaccinia-HSV recombinants expressing gD (Aurelian, L. et al., 1991, Rev.Infect.Dis., 13, S924-S930; Rooney, J.F. et al., 1991, Rev.Infect.Dis., 13, S898-S903; Wachsman, M. et al., 1992, Vaccine, 10, pp.447-454) gB (Rooney, J.F. et al., supra), gL and gH (Browne, H. et al., 1993, J.Gen.Virol., 74, pp.2813-2817) have successfully protected animals from HSV challenge. Vaccination by infection with recombinant adenovirus expressing HSV gB elicits a protective immune response in mice. (Ghiasi, H., supra; McDermott, M.R., 1989, Virology, 169, pp.244-247) It is well documented that anti-gD antibodies can protect against HSV infection whether elicited by immunization with native protein (Long, D. et al., 1984, Infect.Immun., 43, pp.761-764) recombinantly expressed protein (Burke, R.L., supra; Stanberry, L.R. et al., 1987, J.Infect.Dis., 155, pp.914-920; Straus, S.E., supra) peptides derived from gD (Eisenberg, R.J. et al., 1985, J.Virol., 56, pp.1014-1027) or transferred passively (Dix, R.D. et al., 1981, Infect.Immun., 34, pp.192-199; Ritchie, M.H. et al., 1993, Investigative Ophthalmology and Visual Sciences, 34, pp.2460-2468).

Studies by Wolff *et al.* (*supra*) originally demonstrated that intramuscular injection of plasmid DNA encoding a reporter gene results in the expression of that gene in myocytes, at and near the sight of injection. Recent reports demonstrated the successful immunization of mice against influenza by the injection of plasmids encoding influenza A hemagglutinin (Montgomery, D.L. et al., 1993, Cell Biol., 12, pp.777-783), or nucleoprotein (Montgomery, D.L. et al., supra; Ulmer, J.B. et al., 1993, Science, 259, pp.1745-1749). The first use of DNA immunization for a herpes virus has been reported (Cox et al., 1993, J.Virol., 67, pp.5664-5667). Injection of a plasmid encoding bovine herpesvirus 1 (BHV-1) glycoprotein g IV gave rise to anti-g IV antibodies in mice and calves. Upon intranasal challenge with BHV-1, immunized calves showed reduced symptoms and shed substantially less virus than controls.

The ability of HSV glycoprotein D to elicit a protective immune response in mice (Long, D. et al., supra) and guinea pigs (Stanberry, L.R. et al., supra; Stanberry, L.R. et al., 1989, Antiviral.Res., 11, pp.203-214) is well documented.

SUMMARY OF THE INVENTION

To test the efficacy of DNA immunization in the prevention of HSV disease, HSV-2 protein-coding DNA sequences were cloned into the eukaryotic expression vector. This DNA construction elicits an immune response when injected into animals. Immunized animals were infected with HSV to evaluate whether or not direct DNA immunization with the gD gene (or other HSV-2 genes) could protect them from disease. Nucleic acids, including DNA constructs and RNA transcripts, capable of inducing *in vivo* expression of human herpes simplex virus (HSV) proteins upon direct introduction into animal tissues via injection or otherwise are therefore disclosed. Injection of these nucleic acids may elicit immune responses which result in the production of cytotoxic T lymphocytes (CTLs) specific for HSV antigens, as well as the generation of HSV-specific antibodies, which are protective upon subsequent HSV challenge. These nucleic acids are useful as vaccines for inducing immunity to HSV, which can prevent infection and/or ameliorate HSV-related disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Panels A, B, and C

A. Western blot analysis of HSV gD expression by V1J:gD-transfected cells is shown;

1. mock infected Vero cells;
2. HSV-2 186 infected Vero cells (moi=1);
3. HSV-2 Curtis infected Vero cells (moi=1);
4. RD cells transfected with V1J:gD;
5. mock transfected RD cells.

B. Western blot analysis of HSV gB expression by V1JNS:gB-transfected cells is shown.

C. Western blot analysis of HSV ICP27 expression by V1J:ICP27-transfected cells is shown.

Fig. 2. Panels A, B, and C

A. Western blot analysis using sera from HSV PNV-immunized (B - 6.25 ug dose of V1J:gD, C - 50 ug dose of V1J:gD) and sham immunized (A) animals is shown on lysates of BHK cells infected with:

1. HSV-1 KOS;
2. HSV-2 186;
3. HSV-2 Curtis;
4. mock infected.

B. Western blot analysis using sera from HSV PNV gB immunized animals is shown.

Fig. 3. ELISA generated group GMT data is shown for HSV PNV-immunized animals receiving a single injection of vaccine; sera were obtained at 4, 7 and 10 weeks post-immunization.

Fig. 4. Survival of HSV-2 challenged animals following two injections with V1J:gD at 200ug; 100ug; 50 ug; 25ug; 12.5ug; 6.25ug; 3.13ug; 1.56 ug; 0.78 ug; or saline only. Since all animals in the 200ug; 100ug; 25ug; 12.5ug; 6.25ug; and 3.13ug groups survived, they are all represented with a single symbol.

Fig. 5. Survival of HSV-2 challenged animals following one injection with V1J:gD at 50 ug; 16.7 ug; 5.0 ug; 1.67 ug; 0.5 ug; 0.167 ug; 0.05 ug; 0.017 ug; 0.005 ug; or saline only.

Fig. 6. Survival of animals immunized with V1JNS:gB following HSV challenge is shown.

Fig. 7 Survival of animals immunized with V1J:gC following HSV challenge is shown.

Fig. 8 The results of survival, mean days to death, paralysis, and vaginal virus titers in HSV-2 infected guinea pigs is shown.

Fig. 9 Guinea pig vaginal lesion scores are shown.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides polynucleotides which, when directly introduced into a vertebrate *in vivo*, including mammals such as humans, induces the expression of encoded proteins within the animal. As used herein, a polynucleotide is a nucleic acid which contains essential regulatory elements such that upon introduction into a living vertebrate cell, is able to direct the cellular machinery to produce translation products encoded by the genes comprising the polynucleotide. In one embodiment of the invention, the polynucleotide is a polydeoxyribonucleic acid comprising HSV genes operatively linked to a transcriptional promoter. In another embodiment of the invention the polynucleotide vaccine comprises polyribonucleic acid encoding HSV genes which are amenable to translation by the eukaryotic cellular machinery (ribosomes, tRNAs, and other translation factors). Where the protein encoded by the polynucleotide is one which does not normally occur in that animal except in pathological conditions, (i.e. an heterologous protein) such as proteins associated with HSV, the animals'

immune system is activated to launch a protective immune response. Because these exogenous proteins are produced by the animals' own tissues, the expressed proteins are processed by the major histocompatibility system (MHC) in a fashion analogous to when an actual HSV infection occurs. The result, as shown in this disclosure, is induction of immune responses against HSV. Polynucleotides for the purpose of generating immune responses to an encoded protein are referred to herein as polynucleotide vaccines or PNV.

There are many embodiments of the instant invention which those skilled in the art can appreciate from the specification. Thus, different transcriptional promoters, terminators, carrier vectors or specific gene sequences may be used successfully.

The instant invention provides a method for using a polynucleotide which, upon introduction into mammalian tissue, induces the expression, *in vivo*, of the polynucleotide thereby producing the encoded protein. It is readily apparent to those skilled in the art that variations or derivatives of the nucleotide sequence encoding a protein can be produced which alter the amino acid sequence of the encoded protein. The altered expressed protein may have an altered amino acid sequence, yet still elicits antibodies which react with the viral protein, and are considered functional equivalents. In addition, fragments of the full length genes which encode portions of the full length protein may also be constructed. These fragments may encode a protein or peptide which elicits antibodies which react with the viral protein, and are considered functional equivalents.

In one embodiment of this invention, a gene encoding an HSV gene product is incorporated in an expression vector. The vector contains a transcriptional promoter recognized by eukaryotic RNA polymerase, and a transcriptional terminator at the end of the HSV gene coding sequence. In a preferred embodiment, the promoter is the cytomegalovirus promoter with the intron A sequence (CMV-intA), although those skilled in the art will recognize that any of a number of other known promoters such as the strong immunoglobulin, or other eukaryotic gene promoters may be used. A preferred transcriptional

terminator is the bovine growth hormone terminator. The combination of CMVintA-BGH terminator is preferred. In addition, to assist in preparation of the polynucleotides in prokaryotic cells, an antibiotic resistance marker is also optionally included in the expression vector under transcriptional control of a suitable prokaryotic promoter. Ampicillin resistance genes, neomycin resistance genes or any other suitable antibiotic resistance marker may be used. In a preferred embodiment of this invention, the antibiotic resistance gene encodes a gene product for neomycin resistance. Further, to aid in the high level production of the polynucleotide by growth in prokaryotic organisms, it is advantageous for the vector to contain a prokaryotic origin of replication and be of high copy number. Any of a number of commercially available prokaryotic cloning vectors provide these elements. In a preferred embodiment of this invention, these functionalities are provided by the commercially available vectors known as the pUC series. It may be desirable, however, to remove non-essential DNA sequences. Thus, the lacZ and lacI coding sequences of pUC may be removed. It is also desirable that the vectors are not able to replicate in eukaryotic cells. This minimizes the risk of integration of polynucleotide vaccine sequences into the recipients' genome.

In another embodiment, the expression vector pnRSV is used, wherein the rous sarcoma virus (RSV) long terminal repeat (LTR) is used as the promoter. In yet another embodiment, V1, a mutated pBR322 vector into which the CMV promoter and the BGH transcriptional terminator were cloned is used. In a preferred embodiment of this invention, the elements of V1 and pUC19 have been combined to produce an expression vector named V1J. Into V1J or another desirable expression vector is cloned an HSV gene, such as gD, or any other HSV gene which can induce anti-HSV immune responses (antibody and/or CTLs) such as gB, gC, gL, gH and ICP27. In another embodiment, the ampicillin resistance gene is removed from V1J and replaced with a neomycin resistance gene, to generate V1J-neo, into which any of a number of different HSV genes may be cloned for use according to this invention. In yet another embodiment, the vector is

V1Jns, which is the same as V1Jneo except that a unique SfiI restriction site has been engineered into the single KpnI site at position 2114 of V1J-neo. The incidence of SfiI sites in human genomic DNA is very low (approximately 1 site per 100,000 bases). Thus, this vector allows careful monitoring for expression vector integration into host DNA, simply by SfiI digestion of extracted genomic DNA. In a further embodiment, the vector is V1R. In this vector, as much non-essential DNA as possible is "trimmed" to produce a highly compact vector. This vector allows larger inserts to be used, with less concern that undesirable sequences are encoded and optimizes uptake by cells when the construct encoding specific virus genes is introduced into surrounding tissue. The methods used in producing the foregoing vector modifications and development procedures may be accomplished according to methods known by those skilled in the art.

From this work those skilled in the art will recognize that one of the utilities of the instant invention is to provide a system for *in vivo* as well as *in vitro* testing and analysis so that a correlation of HSV sequence diversity with serology of HSV neutralization, as well as other parameters can be made. The isolation and cloning of these various genes may be accomplished according to methods known to those skilled in the art. This invention further provides a method for systematic identification of HSV strains and sequences for vaccine production. Incorporation of genes from primary isolates of HSV strains provides an immunogen which induces immune responses against clinical isolates of the virus and thus meets a need as yet unmet in the field. Furthermore, if the virulent isolates change, the immunogen may be modified to reflect new sequences as necessary.

In one embodiment of this invention, a gene encoding an HSV protein is directly linked to a transcriptional promoter. The use of tissue-specific promoters or enhancers, for example the muscle creatine kinase (MCK) enhancer element may be desirable to limit expression of the polynucleotide to a particular tissue type. For example, myocytes are terminally differentiated cells which do not divide. Integration of foreign DNA into chromosomes appears to require both cell division and protein

synthesis. Thus, limiting protein expression to non-dividing cells such as myocytes may be preferable. However, use of the CMV promoter is adequate for achieving expression in many tissues into which the PNV is introduced.

PNV Construct Summary

HSV and other genes are preferably ligated into an expression vector which has been specifically optimized for polynucleotide vaccinations. Elements include a transcriptional promoter, immunogenic epitopes, and additional cistrons encoding immunoenhancing or immunomodulatory genes, with their own promoters, transcriptional terminator, bacterial origin of replication and antibiotic resistance gene, as described herein. Optionally, the vector may contain internal ribosome entry sites (IRES) for the expression of polycistronic mRNA. Those skilled in the art will appreciate that RNA which has been transcribed *in vitro* to produce multi-cistronic mRNAs encoded by the DNA counterparts is within the scope of this invention. For this purpose, it is desirable to use as the transcriptional promoter such powerful RNA polymerase promoters as the T7 or SP6 promoters, and performing run-on transcription with a linearized DNA template. These methods are well known in the art.

The protective efficacy of polynucleotide HSV immunogens against subsequent viral challenge is demonstrated by immunization with the DNA of this invention. This is advantageous since no infectious agent is involved, no assembly of virus particles is required, and determinant selection is permitted. Furthermore, because the sequence of viral gene products may be conserved among various strains of HSV, protection against subsequent challenge by another strain of HSV is obtained.

The injection of a DNA expression vector encoding gD may result in the generation of significant protective immunity against subsequent viral challenge. In particular, gD-specific antibodies and CTLs may be produced. Immune responses directed against conserved proteins can be effective despite the antigenic shift and drift of the

variable proteins. Because each of the HSV gene products exhibit some degree of conservation among the various strains of HSV, and because immune responses may be generated in response to intracellular expression and MHC processing, it is expected that many different HSV gD PNV constructs may give rise to cross reactive immune responses.

The invention offers a means to induce heterologous protective immunity without the need for self-replicating agents or adjuvants. The generation of high titer antibodies against expressed proteins after injection of viral protein and human growth hormone DNA, [Tang *et al.*, *Nature* **356**, 152, 1992], indicates this is a facile and highly effective means of making antibody-based vaccines, either separately or in combination with cytotoxic T-lymphocyte vaccines targeted towards conserved antigens.

The ease of producing and purifying DNA constructs compares favorably with traditional protein purification, facilitating the generation of combination vaccines. Thus, multiple constructs, for example encoding gD and any other HSV gene also including non-HSV genes may be prepared, mixed and co-administered. Additionally, protein expression is maintained following DNA injection [H. Lin *et al.*, *Circulation* **82**, 2217 (1990); R.N. Kitsis *et al.*, *Proc. Natl. Acad. Sci. (USA)* **88**, 4138 (1991); E. Hansen *et al.*, *FEBS Lett.* **290**, 73 (1991); S. Jiao *et al.*, *Hum. Gene Therapy* **3**, 21 (1992); J.A. Wolff *et al.*, *Human Mol. Genet.* **1**, 363 (1992)], the persistence of B- and T-cell memory may be enhanced [D. Gray and P. Matzinger, *J. Exp. Med.* **174**, 969 (1991); S. Oehen *et al.*, *ibid.* **176**, 1273 (1992)], thereby engendering long-lived humoral and cell-mediated immunity.

The amount of expressible DNA or transcribed RNA to be introduced into a vaccine recipient will depend on the strength of the transcriptional and translational promoters used. The magnitude of the immune response may depend on the level of protein expression and on the immunogenicity of the expressed gene product. In general, an effective dose of about 1 ng to 5 mg, and preferably about 10 µg to 300 µg is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of

administration such as intraperitoneal, intravenous, or inhalation delivery are also suitable. It is also contemplated that booster vaccinations may be provided. Following vaccination with HSV polynucleotide immunogen, boosting with HSV protein immunogens such as the gD, gB, gC, gG, and gH gene products is also contemplated. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of administration of interleukin-12 protein, concurrently with or subsequent to parenteral introduction of the PNV of this invention may be advantageous.

The polynucleotide may be naked, that is, unassociated with any proteins, adjuvants or other agents which affect the recipients' immune system. In this case, it is desirable for the polycucleotide to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, the DNA may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a DNA-liposome mixture, or the DNA may be associated with an adjuvant known in the art to boost immune responses, such as a protein or other carrier. Agents which assist in the cellular uptake of DNA, such as, but not limited to, calcium ions, may also be used. These agents are generally referred to herein as transfection facilitating reagents and pharmaceutically acceptable carriers. Techniques for coating microprojectiles coated with polynucleotide are known in the art and are also useful in connection with this invention. For DNA intended for human use it may be useful to have the final DNA product in a pharmaceutically acceptable carrier or buffer solution. Pharmaceutically acceptable carriers or buffer solutions are known in the art and include those described in a variety of texts such as Remington's Pharmaceutical Sciences.

In another embodiment, the invention is a polynucleotide which comprises contiguous nucleic acid sequences capable of being expressed to produce a gene product upon introduction of said polynucleotide into eukaryotic tissues *in vivo*. The encoded gene product preferably either acts as an immunostimulant or as an antigen capable of generating an immune response. Thus, the nucleic acid sequences in this

embodiment encode a human herpes simplex virus immunogenic epitope, and optionally a cytokine or a T-cell costimulatory element, such as a member of the B7 family of proteins.

There are several advantages of immunization with a gene rather than its gene product. The first is the relative simplicity with which native or nearly native antigen can be presented to the immune system. Mammalian proteins expressed recombinantly in bacteria, yeast, or even mammalian cells often require extensive treatment to insure appropriate antigenicity. A second advantage of DNA immunization is the potential for the immunogen to enter the MHC class I pathway and evoke a cytotoxic T cell response. Immunization of mice with DNA encoding the influenza A nucleoprotein (NP) elicited a CD8+ response to NP that protected mice against challenge with heterologous strains of flu. (Montgomery, D.L. et al., supra; Ulmer, J. et al., supra)

There is evidence that cell-mediated immunity is important in controlling HSV infection [for review see Nash, A.A. et al., 1985, In: The Herpesviruses, Vol.4, Plenum, New York, and Schmidt, D.S. et al., 1992, In: Rouse (ed.), Current Topics In Microbiology And Immunology, Vol.179, Herpes Simplex Virus; Pathogenesis, Immunobiology and Control, Springer-Verlag, Berlin]. While the majority of HSV CTLs isolated from HSV seropositive patients are of the CD4+ type (Schmidt, D.S. et al., 1988, J.Immunol., 140, pp.3610-3616; Tsutsumi, T. et al., 1986, Clin.Exp.Immunol., 66, pp.507-515) CD8+ clones, including one specific for gD, have been isolated. (Torpey, D.J. et al., 1989, J.Immunol., 142, pp.1325-1332; Yasukawa, M. et al., 1989, J.Immunol., 143, pp.2051-2057; Zarling, J.M. et al., 1986, J.Immunol., 136, pp.4669-4673) In mice, cell transfer and depletion experiments suggest that some CD8+ CTLs protect against infection. (Bonneua, R.H. et al., 1989, J.Virol, 63, pp.1480-1484; Nash, A.A. et al., 1987, J.Gen.Virol., 68, pp.825-833) Immunization with gD via infection with recombinant virus vectors (Paoletti, E. et al., 1984, Proc.Natl.Acad. Sci. USA, 81, pp.193-197; Wachsman, M.L. et al., 1987, J.Infect.Dis., 155, pp.1188-1197; Zheng, B. et al., 1993, Vaccine, 11, pp.1191-1198) protects mice from HSV infection. Live virus vectors, like DNA, have the potential for

MHC class I presentation of the immunogen. However, a recent study using infection by an HSV gD-vaccinia recombinant to immunize mice found that protection from challenge was dependent on the delayed type hypersensitivity functions of L3T4+ cells. (Wachsman, M. et al., 1992, Vaccine, 10, pp.447-454) Although, gD-specific CD8+ cells have been isolated from HSV infected mice, their role in limiting infection is unknown. (Johnson, R.M. et al., 1990, J.Immunol., 145, pp.702-710) Work by Koelle *et al.*, suggests that HSV infection of human fibroblasts and keratinocytes may render them unrecognizable to CD8+ CTLs (Koelle, D.M. et al., 1993, J.Clin.Invest., 91, pp.961-968). In natural HSV infection, the role of CD8+ cells in general, and the role of CD8+ response to gD in particular is not resolved.

Since DNA immunization can evoke both humoral and cell-mediated immune responses, its greatest advantage may be that it provides a relatively simple method to survey a large number of viral genes for their vaccine potential. Plasmids expressing HSV-2 glycoproteins B and C also elicit neutralizing antibodies and protect mice from lethal challenge. However, ICP27 which is known to generate a CTL response and to provide some protection in mice immunized by infection with ICP27-vaccinia recombinant virus (Banks, T.A. et al., 1991, J.Virol., 65, pp.3185-3191) did not provide protection from lethal HSV challenge when mice were vaccinated with PNV ICP27 alone. However, ICP27-encoding DNA may be useful as one component of a multi-HSV gene-containing PNV, and it is contemplated that the present invention includes ICP27 as a component of a multivalent HSV PNV.

Immunization by DNA injection also allows, as discussed above, the ready assembly of multicomponent subunit vaccines. Simultaneous immunization with multiple influenza genes has recently been reported. (Donnelly, J. et al., 1994, Vaccines, in press). The inclusion in an HSV vaccine of genes whose products activate different arms of the immune system may also provide thorough protection from subsequent virus challenge.

The following examples are provided to illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1

Vectors for Vaccine Production

A) V1

The expression vector V1 was constructed from pCMVIE-AKI-DHFR [Y. Whang *et al.*, *J. Virol.* **61**, 1796 (1987)]. The AKI and DHFR genes were removed by cutting the vector with EcoR I and self-ligating. This vector does not contain intron A in the CMV promoter, so it was added as a PCR fragment that had a deleted internal Sac I site [at 1855 as numbered in B.S. Chapman *et al.*, *Nuc. Acids Res.* **19**, 3979 (1991)]. The template used for the PCR reactions was pCMVintA-Lux, made by ligating the Hind III and Nhe I fragment from pCMV6a120 [see B.S. Chapman *et al.*, *ibid.*,] which includes hCMV-IE1 enhancer/promoter and intron A, into the Hind III and Xba I sites of pBL3 to generate pCMVIntBL. The 1881 base pair luciferase gene fragment (Hind III-Sma I Klenow filled-in) from RSV-Lux [J.R. de Wet *et al.*, *Mol. Cell Biol.* 7, 725, 1987] was cloned into the Sal I site of pCMVIntBL, which was Klenow filled-in and phosphatase treated.

The primers that spanned intron A are:
5' primer, SEQ. ID:1:
5'-CTATATAAGCAGAG CTCGTTTAG-3'; The 3' primer, SEQ ID:2:
5'-GTAGCAAAGATCTAAGGACGGTGA CTGCAG-3'.

The primers used to remove the Sac I site are:
sense primer, SEQ ID:3:
5-GTATGTGTCTGAAAATGAGC<u>GT</u>GGAGATTGGGCTCGCAC-3'
and the antisense primer, SEQ ID:4:
5'-GTGCGAGCCCAATCTCC<u>AC</u>GCTCATTTTCAGACACA TAC-3'.

The PCR fragment was cut with Sac I and Bgl II and inserted into the vector which had been cut with the same enzymes.

B) V1J Expression Vector

The purpose in creating V1J was to remove the promoter and transcription termination elements from vector V1 in order to place them within a more defined context, create a more compact vector, and to improve plasmid purification yields.

V1J is derived from vectors V1 and pUC18, a commercially available plasmid. V1 was digested with SspI and EcoRI restriction enzymes producing two fragments of DNA. The smaller of these fragments, containing the CMVintA promoter and Bovine Growth Hormone (BGH) transcription termination elements which control the expression of heterologous genes, was purified from an agarose electrophoresis gel. The ends of this DNA fragment were then "blunted" using the T4 DNA polymerase enzyme in order to facilitate its ligation to another "blunt-ended" DNA fragment.

pUC18 was chosen to provide the "backbone" of the expression vector. It is known to produce high yields of plasmid, is well-characterized by sequence and function, and is of small size. The entire *lac* operon was removed from this vector by partial digestion with the HaeII restriction enzyme. The remaining plasmid was purified from an agarose electrophoresis gel, blunt-ended with the T4 DNA polymerase treated with calf intestinal alkaline phosphatase, and ligated to the CMVintA/BGH element described above. Plasmids exhibiting either of two possible orientations of the promoter elements within the pUC backbone were obtained. One of these plasmids gave much higher yields of DNA in *E. coli* and was designated V1J. This vector's structure was verified by sequence analysis of the junction regions and was subsequently demonstrated to give comparable or higher expression of heterologous genes compared with V1.

C) V1Jneo Expression Vector

It was necessary to remove the amp^r gene used for antibiotic selection of bacteria harboring V1J because ampicillin may not be desirable in large-scale fermenters. The amp^r gene from the pUC backbone of V1J was removed by digestion with SspI and Eam1105I restriction enzymes. The remaining plasmid was purified by agarose gel electrophoresis, blunt-ended with T4 DNA polymerase, and then treated with calf intestinal alkaline phosphatase. The commercially available kan^r gene, derived from transposon 903 and contained within the pUC4K plasmid, was excised using the PstI restriction enzyme, purified by agarose gel electrophoresis, and blunt-ended with T4 DNA polymerase. This fragment was ligated with the V1J backbone and plasmids with the kan^r gene in either orientation were derived which were designated as V1Jneo #'s 1 and 3. Each of these plasmids was confirmed by restriction enzyme digestion analysis, DNA sequencing of the junction regions, and was shown to produce similar quantities of plasmid as V1J. Expression of heterologous gene products was also comparable to V1J for these V1Jneo vectors. V1Jneo#3, referred to as V1Jneo hereafter, was selected which contains the kan^r gene in the same orientation as the amp^r gene in V1J as the expression construct.

D) VIJns Expression Vector

An Sfi I site was added to V1Jneo to facilitate integration studies. A commercially available 13 base pair Sfi I linker (New England BioLabs) was added at the Kpn I site within the BGH sequence of the vector. V1Jneo was linearized with Kpn I, gel purified, blunted by T4 DNA polymerase, and ligated to the blunt Sfi I linker. Clonal isolates were chosen by restriction mapping and verified by sequencing through the linker. The new vector was designated V1Jns. Expression of heterologous genes in V1Jns (with Sfi I) was comparable to expression of the same genes in V1Jneo (with Kpn I).

E) V1Jns-tPA

In order to provide an heterologous leader peptide sequence to secreted and/or membrane proteins, V1Jns was modified to include the human tissue-specific plasminogen activator (tPA) leader. Two synthetic complementary oligomers were annealed and then ligated into V1Jn which had been BglII digested. The sense and antisense oligomers were 5'-GATC ACC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC GA-3', SEQ. ID:5:, and 5'-GAT CTC GCT GGG CGA AAC GAA GAC TGC TCC ACA CAG CAG CAG CAC ACA GCA GAG CCC TCT CTT CAT TGC ATC CAT GGT-3', SEQ. ID:6. The Kozak sequence is underlined in the sense oligomer. These oligomers have overhanging bases compatible for ligation to BglII-cleaved sequences. After ligation the upstream BglII site is destroyed while the downstream BglII is retained for subsequent ligations. Both the junction sites as well as the entire tPA leader sequence were verified by DNA sequencing. Additionally, in order to conform with the consensus optimized vector V1Jns (=V1Jneo with an SfiI site), an SfiI restriction site was placed at the KpnI site within the BGH terminator region of V1Jn-tPA by blunting the KpnI site with T4 DNA polymerase followed by ligation with an SfiI linker (catalogue #1138, New England Biolabs). This modification was verified by restriction digestion and agarose gel electrophoresis.

F) pGEM-3-X-IRES-B7

(where X = any antigenic gene) As an example of a dicistronic vaccine construct which provides coordinate expression of a gene encoding an immunogen and a gene encoding an immuno-stimulatory protein, the murine B7 gene was PCR amplified from the B lymphoma cell line CH1 (obtained from the ATCC). B7 is a member of a family of proteins which provide essential costimulation T cell activation by antigen in the context of major histocompatibility complexes I and II. CH1 cells provide a good source of B7 mRNA because they have the phenotype of being constitutively activated and B7 is expressed primarily by activated antigen presenting cells such as B

cells and macrophages. These cells were further stimulated *in vitro* using cAMP or IL-4 and mRNA prepared using standard guanidinium thiocyanate procedures. cDNA synthesis was performed using this mRNA using the GeneAmp RNA PCR kit (Perkin -Elmer Cetus) and a priming oligomer (5'-GTA CCT CAT GAG CCA CAT AAT ACC ATG-3', SEQ. ID:7:) specific for B7 located downstream of the B7 translational open reading frame. B7 was amplified by PCR using the following sense and antisense PCR oligomers: 5'-GGT ACA AGA TCT ACC ATG GCT TGC AAT TGT CAG TTG ATG C-3', SEQ. ID:8:, and 5'-CCA CAT AGA TCT CCA TGG GAA CTA AAG GAA GAC GGT CTG TTC-3', SEQ. ID:9:, respectively. These oligomers provide BglII restriction enzyme sites at the ends of the insert as well as a Kozak translation initiation sequence containing an NcoI restriction site and an additional NcoI site located immediately prior to the 3'-terminal BglII site. NcoI digestion yielded a fragment suitable for cloning into pGEM-3-IRES which had been digested with NcoI. The resulting vector, pGEM-3-IRES-B7, contains an IRES-B7 cassette which can easily be transferred to V1Jns-X, where X represents an antigen-encoding gene.

G) <u>pGEM-3-X-IRES-GM-CSF</u>

(where X = any antigenic gene) This vector contains a cassette analogous to that described in item C above except that the gene for the immunostimulatory cytokine, GM-CSF, is used rather than B7. GM-CSF is a macrophage differentiation and stimulation cytokine which has been shown to elicit potent anti-tumor T cell activities *in vivo* [G. Dranoff *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**, 3539 (1993).

H) <u>pGEM-3-X-IRES-IL-12</u>

(where X = any antigenic gene) This vector contains a cassette analogous to that described in item C above except that the gene for the immunostimulatory cytokine, IL-12, is used rather than B7. IL-12 has been demonstrated to have an influential role in shifting immune responses towards cellular, T cell-dominated pathways as opposed to humoral responses [L. Alfonso *et al.*, *Science*, **263**, 235, 1994].

EXAMPLE 2

Vector V1R Preparation

In an effort to continue to optimize the basic vaccination vector, a derivative of V1Jns, designated V1R, was prepared. The purpose for this vector construction was to obtain a minimum-sized vaccine vector without unneeded DNA sequences, which still retained the overall optimized heterologous gene expression characteristics and high plasmid yields that V1J and V1Jns afford. It was determined from the literature as well as by experiment that (1) regions within the pUC backbone comprising the *E. coli* origin of replication could be removed without affecting plasmid yield from bacteria; (2) the 3'-region of the *kan*^{r} gene following the kanamycin open reading frame could be removed if a bacterial terminator was inserted in its place; and, (3) ~300 bp from the 3'- half of the BGH terminator could be removed without affecting its regulatory function (following the original KpnI restriction enzyme site within the BGH element).

V1R was constructed by using PCR to synthesize three segments of DNA from V1Jns representing the CMVintA promoter/BGH terminator, origin of replication, and kanamycin resistance elements, respectively. Restriction enzymes unique for each segment were added to each segment end using the PCR oligomers: SspI and XhoI for CMVintA/BGH; EcoRV and BamHI for the *kan* ^{r} gene; and, BclI and SalI for the *ori* ^{r}. These enzyme sites were chosen because they allow directional ligation of each of the PCR-derived DNA segments with subsequent loss of each site: EcoRV and SspI leave blunt-ended DNAs which are compatible for ligation while BamHI and BclI leave complementary overhangs as do SalI and XhoI. After obtaining these segments by PCR each segment was digested with the appropriate restriction enzymes indicated above and then ligated together in a single reaction mixture containing all three DNA segments. The 5'-end of the *ori* ^{r} was designed to include the T2 rho independent terminator sequence

that is normally found in this region so that it could provide termination information for the kanamycin resistance gene. The ligated product was confirmed by restriction enzyme digestion (>8 enzymes) as well as by DNA sequencing of the ligation junctions. DNA plasmid yields and heterologous expression using viral genes within V1R appear similar to V1Jns. The net reduction in vector size achieved was 1346 bp (V1Jns = 4.86 kb; V1R = 3.52 kb).

PCR oligomer sequences used to synthesize V1R (restriction enzyme sites are underlined and identified in brackets following sequence):

(1) 5'-GGT ACA <u>AAT ATT</u> GG CTA TTG GCC ATT GCA TAC G-3' [SspI], SEQ.ID:10:,
(2) 5'-CCA CAT <u>CTC GAG</u> GAA CCG GGT CAA TTC TTC AGC ACC-3' [XhoI], SEQ.ID:11:
(for CMVintA/BGH segment)

(3) 5'-GGT ACA <u>GAT ATC</u> GGA AAG CCA CGT TGT GTC TCA AAA TC-3'[EcoRV], SEQ.ID:12:
(4) 5'-CCA CAT <u>GGA TCC</u> G TAA TGC TCT GCC AGT GTT ACA ACC-3' [BamHI], SEQ.ID:13:
(for kanamycin resistance gene segment)

(5) 5'-GGT ACA <u>TGA TCA</u> CGT AGA AAA GAT CAA AGG ATC TTC TTG-3'[BclI], SEQ.ID:14:,
(6) 5'-CCA CAT <u>GTC GAC</u> CC GTA AAA AGG CCG CGT TGC TGG-3' [SalI], SEQ.ID:15:
(for *E. coli* origin of replication)

EXAMPLE 3

Cells, Viruses and Cell culture

VERO, BHK-21, and RD cells were obtained from the ATCC. Virus was routinely prepared by infection of nearly confluent VERO or BHK cells with a multiplicity of infection (m.o.i.) of 0.1 at 37°C in a small volume of medium without fetal bovine serum (FBS). After one hour, virus inoculum was removed and cultures were re-fed with high glucose DMEM supplemented with 2% heat-inactivated FBS, 2mM L-glutamine, 25mM HEPES, 50 U/ml penicillin and 50 µg/ml streptomycin. Incubation was continued until cytopatic effect was extensive: usually 24 to 48 hours. Cell associated virus was collected by centrifugation at 1800 X g 10 minutes 4°C. Supernantant virus was clarified by centifugation at 640 X g for 10 minutes 4°C.

EXAMPLE 4

Cloning and DNA preparation

HSV-2 (Curtis) DNA for use as PCR template was prepared from nucleocapsids isolated from infected VERO cells. (Denniston, K.J. et al., 1981, Gene, 15, pp.365-378) Synthetic oligomers corresponding to 5' and 3' end flanking sequences for the HSV2 gB, gC, gD, or ICP27 genes, containing Bgl II restriction recognition sites (Midland Certified Reagent Company; Midland, Texas) were used at 20 pmoles each. A 1.1kb fragment encoding the gD gene was amplified by PCR (Perkin Elmer Cetus, La Jolla) according to the maufacturer's specifications except that a deaza dGTP:dGTP ratio of 1:4 was used in place of dGTP and the buffer was supplemented to 3 mM Mg Cl_2. HSV-2 genomic DNA template was used at 100 ng/100 µl reaction. The PCR amplified fragments were restricted with Bgl II and ligated to the Bgl II digested, dephosphorylated vector V1J (Montgomery, D.L. et al., supra). *E. coli* DH5α (BRL-Gibco, Gaithersburg, Md.) was transformed according to the manufacturer's specifications. Ampicillin resistant colonies were screened by hybridization with the ^{32}P labeled 3' PCR primer. Candidate

plasmids were characterized by restriction mapping and sequencing of the vector-insert junctions using the Sequenase DNA Sequencing Kit. version 2.0 (United States Biochemical). In a similar manner, a 2.7Kb fragment encoding the gB gene; a 1.5Kb fragment encoding the gC gene; and a 1.6Kb fragment encoding the ICP27 gene were also PCR amplified. Independently derived isolates were identified and characterized for the presence of the correct DNA construct containing either the gB, gC, gD, or ICP27 gene.

Large scale DNA preparation was essentially as described (Montgomery, D.L. et al., supra) except that 800 ml cultures were grown for 24 to 48 hours and for some experiments DNA was purified by a single CsCl-EtBr isopyncnic density centifugation.

The plasmid constructions were characterized by restriction mapping and sequence analysis of the vector-insert junctions. Results were consistent with published HSV-2 strain G (Lasky, L.A. et al., 1984, DNA, 3, pp.23-29) sequence data and showed that initiation and termination codons were intact for each construct.

EXAMPLE 5

Expression of HSV-2gB, gC, gD and ICP27 proteins from V1J plasmids

Rhabdomyosarcoma cells (ATCC CCL136) were planted one day before use at a density of 1.2 X10^6 cells per 9.5 cm^2 well in six-well tissue culture clusters in high glucose DMEM supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 25 mM HEPES, 50 U/ml penicillin and 50 μg/ml streptomycin. (All from BRL-Gibco) Phenol : chloroform extracted cesium chloride purified plasmid DNA was precipitated with calcium phosphate using Pharmacia CellPhect reagents according to the kit instructions except that 5 - 15 μg is used for each 9.5 cm^2 well of RD cells. Cultures were glycerol shocked six hours post addition of calcium phosphate-DNA precipate; after refeeding, cultures were incubated for two days prior to harvest.

Lysates of transfected cultures were prepared in 1X RIPA (0.5% SDS, 1.0% TRITON X-100, 1% sodium deoxycholate, 1mM

EDTA, 150mM NaCl, 25 mM TRIS-HCl pH 7.4) supplemented with 1µM leupeptin, 1µM pepstatin, 300nM aprotinin, and 10µM TLCK, and sonicated briefly to reduce viscosity. Lysates were resolved by electrophoresis on 10% Tricine gels (Novex) and then transferred to nitrocellulose membranes. Immunoblots were processed with HSV-2 convalescent mouse sera and developed with the ECL detection kit (Amersham).

Expression of HSV gD from V1J:gD was demonstrated by transient transfection of RD cells. Lysates of V1J:gD-transfected or mock transfected cells were fractionated by SDS PAGE and analyzed by immunoblotting. Figure 1A shows that V1J:gD transfected RD cells express an immunoreactive protein with an apparent molecular weight of approximately 55 K. Lysates from HSV-2 (Curtis), HSV-2 (186), or mock-infected Vero cells are included for comparison. The identical migrations of cloned gD and the authentic protein from infected cells indicates that the protein is ful- length, and is processed and glycosylated similarly to that of gD in HSV-infected cells. Indirect immunofluorescence of fixed V1J:gD transfected cells showed a diffuse cytoplasmic signal.

Expression of HSV gB from V1JNS:gB was demonstrated by transient transfection of RD cells. Lysates of V1JNS:gB-transfected or mock transfected cells were fractionated by SDS PAGE and analyzed by immunoblotting. Figure 1B shows that V1JNS:gB transfected RD cells express an immunoreactive protein with an apparent molecular weight of approximately 140 k. Lysates from HSV-2 (Curtis), HSV-2 (186), or mock infected Vero cells are included for comparison. The similar migrations of cloned gB and the authentic protein from infected cells shows that the protein is full-length. Indirect immunofluorescence of fixed V1JNS:gB transfected cells showed a membrane-associated punctate signal.

Expression of HSV gC from V1J:gC was demonstrated by transient transfection of RD cells. Indirect immunofluorescence of fixed V1J:gC transfected cells showed primarily a diffuse cytoplasmic signal.

Expression of ICP27 was demonstrated by transient transfection of RD cells, followed by Western blot analysis. A mouse monoclonal antibody specific for ICP27 detected a protein of about 60 k Da, which is consistent with the major immunoreactive protein in HSV 2 infected cells (Figure 1C).

EXAMPLE 6

Immunization with PNV and detection of anti-HSV antibodies

Five- to six-week-old female BALB/c mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of 5 mg ketamine HCl (Aveco, Fort Dodge, IA) and 0.5 mg xylazine (Mobley Corp., Shawnee, KS.) in saline. The hind legs were shaved with electric clippers and washed with 70% ethanol. Animals were injected with a total of 100 µl of DNA suspended in saline: 50 µl each leg.

The ability of V1J:gD DNA to elicit an immune response to HSV gD was first examined in a titration experiment. Groups of ten mice received i.m. injections of DNA in a dose range from 200 µg to 0.78 µg (8 two-fold dilutions) or were sham immunized with saline. Sera, obtained four and six weeks post immunization, were analyzed by ELISA. For the ELISA, HSV-2 glycoprotein was diluted to 5 µg/ml in 50 mM carbonate buffer pH 9.5. Nunc Maxi-sorb flat bottom 96-well plates were coated at 4°C, overnight with 100 µl per well of HSV glycoproteins. Plates were washed four times with PBS pH 7.2 and nonspecific reactivity was reduced with blocking and dilution buffer, 20 mM TRIS-HCl pH 7.5, 137 mM NaCl, 2.7 mM KCl, 0.5% gelatin, 0.05% Tween 20 for one hour at room temperature. Serial dilutions of mouse sera were added, and plates were incubated one hour at room temperature. Plates were washed four times with PBS and once with distilled water prior to the addition of alkaline phosphatase-labeled goat anti mouse IgG (Boehringer Mannheim, Indianapolis, IN) and incubated

for one hour at room temperature. Excess secondary antibody was removed with four PBS washes followed by one distilled water wash. The ELISA was developed with the addition of 100 µl per well of 1 mg/ml p-nitrophenylphosphate in 10% diethanolamine pH 9.8 100µg/ml MgCl•6 H_2O at 37°C. Absorbance was read at 405nm and serum dilutions were scored as positive if the OD405 was greater than the mean plus three standard deviations of the same dilution of the saline control sera. By four weeks the majority of animals receiving ≥ 6.25 µg of DNA were seropositive. At doses lower than 6.25 µg, fewer animals had seroconverted, however even at the lowest dose some animals were ELISA positive. None of the saline injected control animals were positive. At six weeks a majority of the animals had become seropositive.

At seven weeks, the animals were re-immunized with the same doses of DNA (or saline) used in the initial injections. Sera were obtained at ten weeks (three weeks after the second injection) and endpoint titers were determined by ELISA. The results are summarized in Table 1. By ten weeks, 93% of the DNA injected mice were seropositive. Even at the 0.78 µg dose, eight of the nine animals were positive.

Table 1

Seroconversion of mice immunized with V1J:gD DNA[a]

DNA dose (μg)	no. seropositive/no. immunized	ELISA GMT[b]
200	9/9	31,808
100	10/10	44,904
50	9/10	8,027
25	8/8	13,512
12.5	10/10	14,199
6.25	10/10	16,016
3.13	10/10	9,054
1.56	7/10	360
0.78	8/9	4,641
saline	0/10	10

a - Mice were immunized at weeks 0 and 7 with the indicated amount of DNA. Sera were obtained at 10 weeks and assayed as described herein.
b - For purposes of calculating the GMT, sera negative at the lowest dilution tested (1:30), were assigned a value equal to one dilution less, i.e. 1:10.

To confirm that the ELISA reactivity was due to anti-gD antibodies, several high ELISA titer sera were characterized by their reactivity with immunoblots of HSV or mock-infected cell lysates. Figure 2A illustrates that sera from V1J:gD immunized mice react specifically with a single HSV encoded protein with an electrophoretic mobility consistent with that of HSV gD. Taken together, these data show that i.m. injection of mice with V1J:gD DNA results in the expression of gD epitopes and the development of an immune response to gD protein.

To extend these results and to establish the minimal effective PNV dose, V1J:gD was titrated further in an experiment where animals were immunized only once. Groups of mice were injected with V1J:gD DNA ranging from 5 ng to 50 μg. Sera collected at four, seven, and ten weeks post immunization were assayed by ELISA; the data are summarized in Figure 3.

This titration reveals a threshold of response of about 0.5 μg DNA. While a few animals receiving lower amounts of DNA were seropositive by ELISA, the positive response was transient and occurred only at the lowest serum dilution. At DNA doses of ≥ 1.67 μg, more than 90% of animals seroconverted by four weeks and remained positive at seven and ten weeks.

Increases in antibody titers of individual animals with time are reflected by increases in the group GMTs seen in Figure 3. At doses ≥ 0.5 μg, the GMT rises sharply between four and seven weeks. Between seven and ten weeks, the titers increase or remain constant in all but one case. The ten-week ELISA titers of animals receiving ≥ 0.5 μg are similar to those attained in the previous experiment where a second DNA immunization was given. The amounts of PNV required to provoke an immune response was as much as 100-fold less than published reports using similar vectors. (Robinson <u>et</u> <u>al</u>., 1993, Vaccine, <u>11</u>, pp.957-960; and Fynan <u>et</u> <u>al</u>., 1993, Proc.Natl.Acad.Sci. USA, <u>90</u>, pp.11478-11482)

In order to establish a standardized protocol, the effectiveness of one- and two-dose immunization was compared. In the two-dose experiment, we found no significant differences in protection at the highest (200 μg) dose and the lowest (0.78 μg) dose. When the titration was extended in the single-dose experiment, a dose response for ELISA GMT was observed, showing efficiency of seroconversion and protection. The threshold for these responses was about 0.5 μg. However, seroconversion does occur with as little as 50 ng of DNA. In general, titers continued to rise through ten weeks after a single

injection and in the two-dose experiment there was no obvious boost in titers after the second injection. Finally, at 50 μg of DNA, the only dose common to both experiments, there were no significant differences between one and two doses.

Similar analyses as set forth above were done for the PNV constructions containing the HSV genes gB and gC. Mice (10 mice per group) were immunized as described above with DNA containing the HSV gB gene or DNA containing the HSV gC gene. Serum was collected and analyzed for the presence of anti-gB or anti-gC antibodies in the ELISA described above. The ELISA data for gB antibodies are shown in Table 2, and demonstrates that mice immunized with V1JNS:gB were seropositive for anti-gB antibodies.

TABLE 2

10 WEEK SERA ELISA gB

Treatment	weeks	GMT	SEM (range)
saline	0	3	3-3
1ug	0	3	3-3
1ug	0, 7	3	3-3
3ug	0	6	4-10
3ug	0, 7	24	8-68
10ug	0	48	13-170
10ug	0, 7	150	38-595
30ug	0	300	87-1034
30ug	0, 7	39	10-150
100ug	0	378	69-2062
100ug	0, 7	7536	1893-30,000

The ELISA data for gC antibodies are shown in Table 3, and demonstrates that mice (five mice per group) immunized with V1J:gC were seropositive for anti-gC antibodies.

TABLE 3

10 WEEK SERA ELISA gC

Treatment	weeks	GMT	SEM (range)
saline	0	10	10-10
10ug	0, 7	4642	2154-10,000
100ug	0, 7	3162	1440-6943

To confirm that the ELISA reactivity for gB was due to anti-gB antibodies, several high ELISA titer sera were characterized by their reactivity with immunoblots of HSV or mock infected cell lysates. Figure 2B illustrates that sera from V1JNS:gB immunized mice reacts specifically with a single HSV encoded protein with an electrophoretic mobility consistent with that of HSV gB. Taken together, these data show that i.m. injection of mice with HSV PNV results in the expression of HSV epitopes and the development of an immune response to those HSV proteins.

EXAMPLE 7

HSV Neutralization

Mouse sera were heat-inactivated at 56°C 30 minutes prior to serial dilution in DMEM, 2% heat inactivated FBS and then 50 μl of each dilution was delivered to duplicate wells in a sterile polypropylene 96 deep well plate (Marsh Biomedical, Rochester, NY.). HSV-1 or HSV-2 stocks were diluted to 4,000 pfu/ml, 50 μl of virus were then added to each sample well and the plate was incubated overnight at 4°C. Guinea pig complement (Cappel) was diluted 1:4 in DMEM, 2% heat inactivated FBS and 50 μl were added to each sample well. After a one hour incubation at 37°C, 100 μl of serum free medium was added to each well and each reaction mixture was used to infect confluent VERO cells in 12-well cluster plates. (Costar) Neutralized virus samples were adsorbed for one hour at 37°C. Inocula were gently aspirated and monolayers were

overlaid with 1 ml 0.5% carboxymethylcellulose 1X MEM 5% heat inactivated FBS 10 mM l-glutamine, 25U/ml penicillin, 25 μg/ml streptomycin, 12.5 mM HEPES. Plates were incubated at 37°C for 48 hours. Overlays were removed and cell monolayers were stained with 1% basic fuchsin, 50% methanol 10% phenol. Plaques were counted and the neutralization titer was determined as the serum dilution which yielded a 50% reduction in plaque number when compared to sera from sham-immunized mice.

To determine whether the anti-gD antibodies might be biologically active, selected high-titer ten-week sera from mice immunized at zero and seven weeks were assayed for HSV-2 neutralizing activity. The results of plaque reduction assays are in Table 4. Sera from V1J:gD immunized mice neutralized not only HSV-2 (Curtis) but also HSV-2 (186). Furthermore, at least some of the sera contain type common neutralizing antibodies as shown by their neutralization of HSV-1 (KOS) infectivity. Although the neutralizing titers were low in some cases, these results encouraged us to see if these anti-gD antibodies could protect the animals against a lethal HSV challenge.

Ten week sera from all animals immunized with ≥0.5 μg V1J:gD in the single-dose experiment were also tested in an HSV-2 plaque reduction assay. Twenty-nine of the forty-nine sera assayed were positive: >50% plaque reduction at a 1:10 dilution. At the 16.7 and 50 μg dose level, nine of ten sera from each group were neutralization positive.

TABLE 4

animal no.	HSV-2 Curtis neutralization (serum dilution) 1:10	1:100	1:1000	DNA dose (μg)	ELISA titer
4353 a	+	+	-	3.13	≥ 33,333
4354	+	+	-	3.13	33,333
4362	+	+	-	6.25	≥ 33,333
4363 b	+	-	-	6.25	≥ 33,333
4371	+	+	-	12.5	≥ 33,333
4391	+	+	+	50	1000
4392 a	+	+	+	50	10,000
4393	-	-	-	50	333
4395	-	-	-	50	≤ 100
4396 b	+	+	-	50	≥ 33,333
4397	+	-	-	50	≥ 33,333
4398	+	+	+	50	≥ 33,333
4399	+	+	+	50	≥ 33,333
4400	+	+	-	50	3,333
4405 b	+	+	+	100	100,000

a - neutralizes HSV-1 KOS at 1:100.
b - neutralized HSV-2 186 at 1:100.

EXAMPLE 8

HSV Challenge

Stocks of challenge virus were prepared by infection of confluent VERO monolayers with HSV-2 Curtis as described above. Clarified supernatant virus was titered on VERO cells and aliquots were stored at -70°C. Animals were infected by i.p. injection with 0.25 ml of virus stock and then observed for three weeks. Survival data were analyzed using the log-rank test (McDermott et al., 1989, Virology, 169,

pp.244-247) in the SAS® procedure LIFETEST. Differences in probability ≤ 0.001 were judged highly significant.

Eleven weeks after the initial DNA injection, mice immunized with two doses of V1J:gD were challenged by i.p. injection of $10^{5.7}$ p.f.u. of HSV-2 (Curtis) and observed for 21 days. Survival data are in Figure 4. It is readily apparent that animals immunized with as little 0.78 μg of V1J:gD were significantly protected from lethal infection. Of the three immunized animals that died, two were seronegative by ELISA at ten weeks. A few of the surviving animals did show signs of transient illness including failure to groom, failure to thrive, or a hunched posture. While the level of protection from death achieved at every dose of DNA was significant ($p< 0.01$), these symptoms suggest some break-through infection occurred. Analysis of sera obtained from convalescent animals were characterized by their reaction in immunoblots of HSV-2 infected Vero cell lysates. In some cases, a serum recognized only gD and in others, the serum reacted with many HSV proteins. These results are consistent with at least some of the mice having experienced HSV infection.

Animals immunized with a single DNA injection were challenged as described above. Survival data are presented in Figure 5. Statistically significant ($p < .001$) protection against death was obtained in groups of animals receiving ≥1.67 μg V1J:gD. This survival dose response is similar to that seen for ELISA titer (Fig. 3). As was seen in the two-dose experiment, a few surviving animals displayed transient signs of illness during the observation period. Surviving animals immunized with higher doses of DNA (16.7 and 50 μg) remained sleek and healthy-looking throughout the observation period.

Animals immunized with PNV constructs containing HSV gB or gC genes were also challenged with a lethal dose of HSV as described above for gD. Survival data for animals immunized with V1JNS:gB are shown in Figure 6, and survival data for animals immunized with V1J:gC are shown in Figure 7 demonstrating that protection from death was obtained.

These results demonstrate the potential for direct DNA immunization in the prevention of HSV infection. Using glycoprotein gD as a model, it was found that a single i.m. injection of as little as 0.5 μg V1J:gD DNA elicited a neutralizing antibody response to gD that afforded statistically significant protection against lethal HSV challenge. Immunization with as little as 3.13 μg of the DNA in a two-dose regimen protected all animals from death.

EXAMPLE 9

Vaccination of Guinea Pigs with HSV PNV

Hartley strain guinea pigs (Harlan Sprague Dawley Labs, Indianapolis, IN) weighing approximately 200-250 grams each were vaccinated intramuscularly 0.1 ml in the right thigh and 0.1 ml in the left thigh at 11 and 4 weeks prior to virus challenge. Fresh solutions of the vaccine and placebo were sent to us for each vaccination.

In order to determine HSV-2 antibody production in the animals, the guinea pigs were bled 5 weeks after the first vaccination and 2 weeks after the second vaccination. Blood (0.6-1 ml per animal) was obtained by toe clipping. The blood was collected in microseparation tubes (Becton Dickinson), and was later centrifuged at 1000 x g for 10 minutes to separate the serum.

The sera collected from the guinea pigs was analyzed for the presence of anti-HSV antibodies using the ELISA set forth in Example 6. The results are shown in Table 5.

TABLE 5

10 WEEK GUINEA PIG SERA ELISA gD

Treatment	GMT	SEM (range)
saline	3	3-3
10ug DNA	19	8-43
100ug DNA	599	277-1295

At the time of infection, the guinea pigs weighed 600-700 grams each. They were infected intravaginally with herpes simplex virus type 2 (HSV-2), E194 strain. This was accomplished in a 3-step process. First, the vagina of each animal was swabbed for 5 seconds with a cotton tip applicator dipped in 0.1 N NaOH. This treatment irritates the vaginal area so that the infection takes better. Approximately 45-60 minutes later each vagina was dry swabbed for 5 seconds. Then an applicator dipped in virus medium (about 5 x 10^6 plaque forming units of HSV-2 per ml) was used to swab each guinea pig for 20 seconds. The swabs were gently and slowly twisted back and forth during the time they were in place.

Lesion scores in infected animals were determined daily at day 2-15 post infection. A score of 1+ indicates about 25% of the anal-vaginal area was affected (usually by redness immediately around the vagina); 2+ indicates 50% of the anal-vaginal area affected; 3+ indicates 75% affected; and 4+ indicates 100% affected. Because some of the animals went on to die, the lesion score near the time of death carried through to the end of the 15 days. If this were not done, average lesion scores would appear to go down since the most affected animals died off.

Deaths were recorded daily for 21 days. The mean day of death calculation took into account only guinea pigs that die. Numbers of animals with hind limb paralysis were noted throughout the infection. Vaginal virus titers were made by titration of virus obtained from vaginal swabs at 2, 4 and 6 days after virus inoculation. The swabs were placed into tubes containing 1 ml of cell culture medium. The titration of these samples was conducted in Vero cells in 96-well plates. Calculation of virus titer was made by the 50% endpoint dilution method of Reed L. J. and Muench M., *Am. J. Hyg.* **27**, 493-498 (1938). Virus titers were expressed as $\log_{10}$ cell culture infectious doses per ml.

Statistical interpretations of survival (Fisher exact test), mean days to death (Mann-Whitney U test), paralysis (Fisher exact test), vaginal virus titers (Mann-Whitney U test), and vaginal lesion scores (Mann-Whitney U test) were made by two-tailed analyses.

Figure 8 shows the results of survival, mean days to death, paralysis, and vaginal virus titers in HSV-2 infected guinea pigs. The high dose of vaccine prevented mortality and reduced vaginal virus titers on days 2 and 4 relative to the placebo control. The high dose of vaccine significantly prevented paralysis in these animals. The low dose of vaccine also reduced the above parameters.

Table 7 shows daily vaginal lesion scores for the experiment. Both the high and low doses of the vaccine caused significant reductions in vaginal lesion severity from days 3 through 15 of the infection compared to the placebo group. The results in Table 7 are presented graphically in Figure 9.

These results clearly indicate that the vaccine was protective in guinea pigs infected with HSV-2, and that the high dose of vaccine was more active than the low dose. The high dose of vaccine was not able to completely block the infection, since virus was recovered from vaccinees and a low grade of vaginal lesion development occurred. Nevertheless, the degree of protection afforded by the vaccine at this dose was substantial. The results of the antibody studies correlate with antiviral protection.

The vaccine administered intramuscularly at two different doses to guinea pigs 11 and 4 weeks prior to intravaginal HSV-2 challenge significantly protected animals from the disease. The high dose of vaccine was more effective than the low dose. The vaccine appears to be safe in the animals.

TABLE 7

Day[b]	Vaccine[a], 10 µg	Vaccine[a], 100 µg	Placebo
3	0.2 ± 0.2	0.1 ± 0.2	0.3 ± 0.3
4	0.2 ± 0.3*	0.3 ± 0.4*	0.7 ± 0.3
5	0.4 ± 0.4**	0.4 ± 0.4**	1.6 ± 1.1
6	1.0 ± 1.0	0.5 ± 0.6*	1.9 ± 1.4
7	1.5 ± 1.2*	0.6 ± 0.7***	3.0 ± 1.3
8	1.0 ± 1.0**	0.5 ± 0.5***	3.2 ± 1.3
9	0.6 ± 0.7***	0.6 ± 0.7***	2.7 ± 1.4
10	0.5 ± 0.5***	0.4 ± 0.5***	2.5 ± 1.1
11	0.5 ± 0.5***	0.3 ± 0.4***	2.3 ± 0.8
12	0.5 ± 0.4***	0.3 ± 0.4***	2.4 ± 0.7
13	0.5 ± 0.5***	0.5 ± 0.6***	2.3 ± 0.8
14	0.5 ± 0.6***	0.6 ± 0.6***	2.3 ± 0.6
15	0.5 + 0.6***	0.7 + 0.7***	2.0 + 0.7
Grand Avg. (Days 3-15)	0.6 + 0.4***	0.4 + 0.2***	2.1 + 0.8

a Intramuscular vaccinations were given 11 and 4 weeks before virus challenge.

b After virus inoculation.

* P<0.05, **P<0.01, ***P<0.001.

EXAMPLE 10

To determine whether mice vaccinated intramuscularly with PNV HSV would produce mucosal HSV-specific antibodies, mice were vaccinated with 12.5 or 1.56 µg of V1JNS:gD. Vaginal fluid was collected by swab and the antibodies were eluted from the swab using

phosphate buffered saline. The eluant was analyzed for the presence of IgG and IgA, specific for HSV-2 protein. The ELISA was performed as described above except that commercially available antibodies specific for mouse IgG (Boehringer) and specific for mouse IgA (Seralab) were used to detect the presence of HSV-specific IgG and IgA in the mouse vaginal samples. The results for IgG are shown in Table 8; IgA was not detected in any animal.

TABLE 8

Animal No.	ELISA Development Time (minutes) 30	60
1031	<0.1	<0.1
1032	0.1	0.1
1033	0.01	0.01
1035	<0.1	0.1
1037	<0.1	<0.1
1038	<0.1	<0.1
1039	<0.1	<0.1
1040	<0.1	<0.1

[a] - injected with saline
[b] - injected with 1.56 μg V1JNS:gD

The results demonstrate the presence of mucosal IgG specific for HSV-2 in mice vaccinated with V1J:gD.

SEQUENCE LISTING

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTATATAAGC AGAGCTCGTT TAG 23

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTAGCAAAGA TCTAAGGACG GTGACTGCAG 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTATGTGTCT GAAAATGAGC GTGGAGATTG GGCTCGCAC 39

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGCGAGCCC AATCTCCACG CTCATTTTCA GACACATAC 39

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCACCATG GATGCAATGA AGAGAGGGCT CTGCTGTGTG CTGCTGCTGT GTGGAGCAGT 60

CTTCGTTTCG CCCAGCGA 78

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCTCGCTG GGCGAAACGA AGACTGCTCC ACACAGCAGC AGCACACAGC AGAGCCCTCT 60

CTTCATTGCA TCCATGGT 78

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTACCTCATG AGCCACATAA TACCATG 27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGTACAAGAT CTACCATGGC TTGCAATTGT CAGTTGATGC 40

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCACATAGAT CTCCATGGGA ACTAAAGGAA GACGGTCTGT TC 42

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGTACAAATA TTGGCTATTG GCCATTGCAT ACG 33

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCACATCTCG AGGAACCGGG TCAATTCTTC AGCACC 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTACAGATA TCGGAAAGCC ACGTTGTGTC TCAAAATC 38

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCACATGGAT CCGTAATGCT CTGCCAGTGT TACAACC 37

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGTACATGAT CACGTAGAAA AGATCAAAGG ATCTTCTTG 39

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCACATGTCG ACCCGTAAAA AGGCCGCGTT GCTGG 35

WHAT IS CLAIMED IS:

1. A polynucleotide which induces anti-HSV antibodies or protective immune responses upon introduction into vertebrate tissue, wherein said polynucleotide comprises one or more genes encoding one or more HSV proteins or functional equivalents thereof, said genes being operably linked to a transcription promoter.

2. The polynucleotide of Claim 1, wherein said gene encodes an HSV protein selected from a group consisting of gB, gC, gD, gH, gL, ICP27, and functional equivalents thereof.

3. A method for inducing immune responses in a vertebrate against HSV epitopes which comprises introducing between 1 ng and 5 mg of a polynucleotide according to Claim 1 into a tissue of a vertebrate.

4. A vaccine for inducing immune responses against HSV which comprises the polynucleotide of Claim 1 and a pharmaceutically acceptable carrier.

5. A method for inducing immune responses against HSV which comprises introducing into a tissue of a vertebrate one or more isolated and purified HSV genes eliciting an immune response which prevents HSV infection and/or ameliorates HSV disease.

6. A polynucleotide comprising:

a) a eukaryotic transcription promoter;
b) an open reading frame operably linked to said promoter encoding one or more HSV epitopes, and a translation termination signal; and

c) optionally containing one or more operably linked IRES, one or more open reading frames encoding one or more additional genes, and one or more transcription termination signals.

7. The polynucleotide of Claim 6 wherein said additional genes of c) are immunomodulatory or immunostimulatory genes selected from a group consisting of GM-CSF, IL-12, interferon, and a member of the B7 family of T-cell costimulatory proteins.

8. The polynucleotide of Claim 6 wherein said HSV gene of a) encodes an HSV protein selected from a group consisting of gB, gC, gD, gH, gL, ICP27, and functional equivalent thereof.

9. The polynucleotide of Claim 6 wherein said additional genes of c) are HSV genes selected from a group consisting of gB, gC, gD, gH, gL, ICP27, and functional equivalent thereof.

10. A method of treating a patient in need of such treatment with a ploynucleotide which induces anti-HSV antibodies or protective immune responses upon introduction into vertebrate tissue, wherein said polynucleotide comprises a gene encoding one or more HSV proteins or functional equivalents thereof, said gene being operably linked to a transcription promoter.

11. The method of claim 10 wherein said polynucleotide comprises a gene encoding one or more HSV proteins selected from a group consisting of gB, gC, gD, gH, gL, ICP27, and functional equivalents thereof.

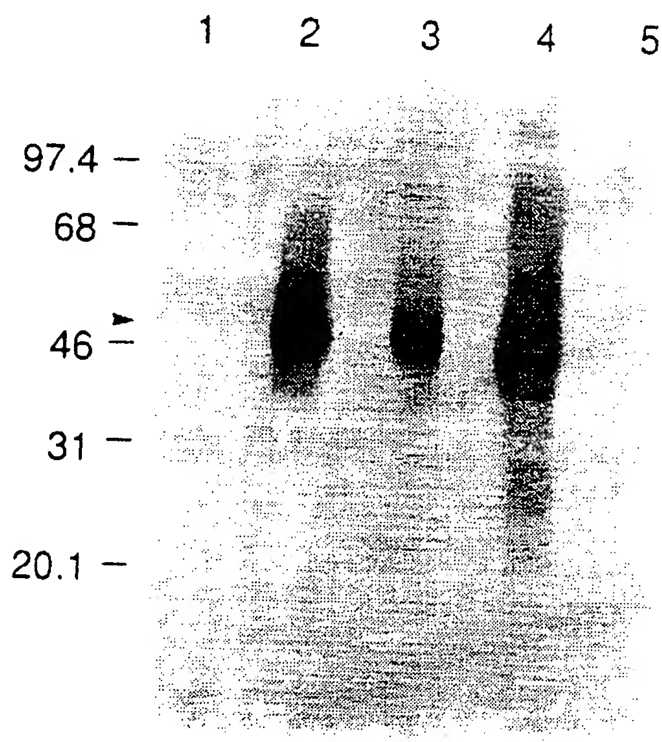


FIG. 1A

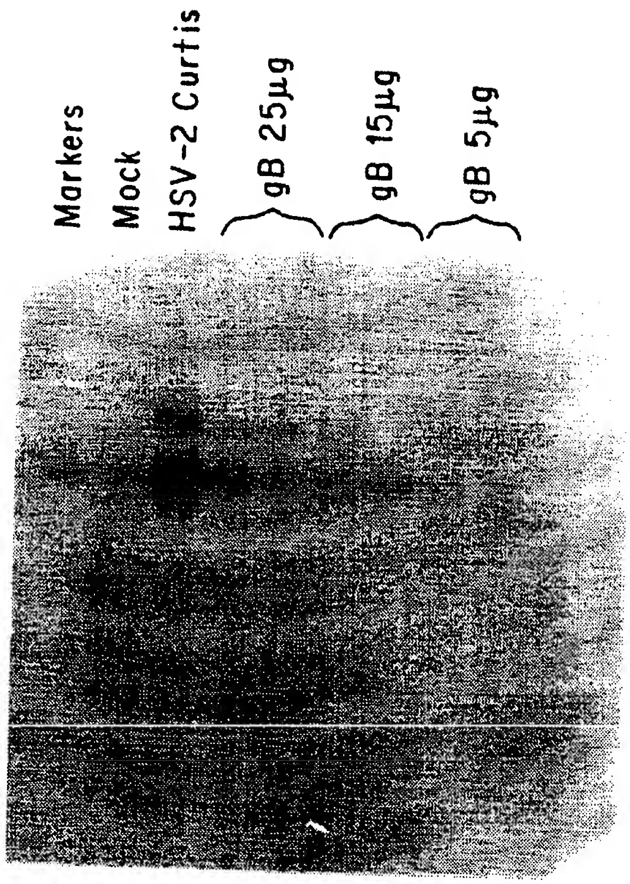
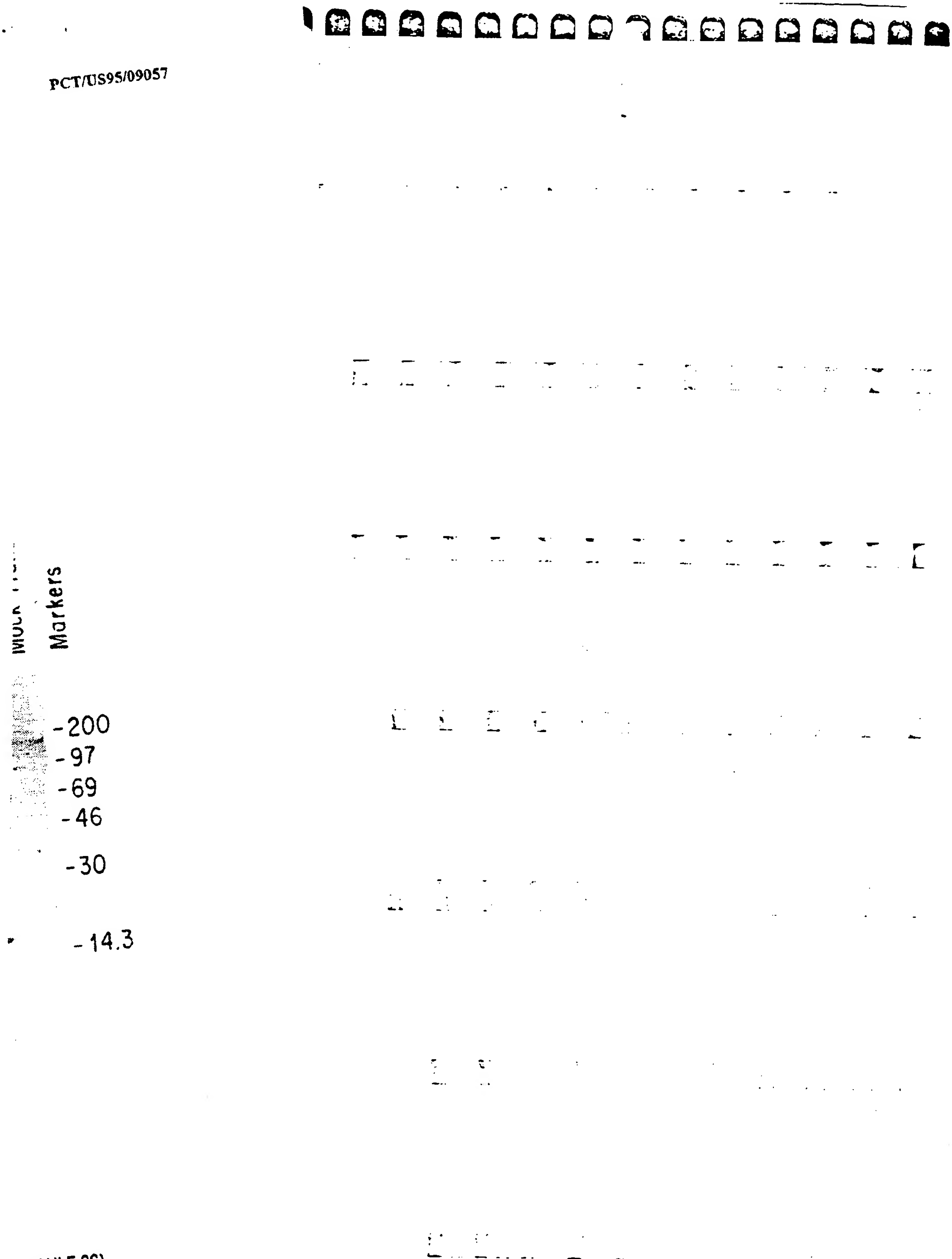
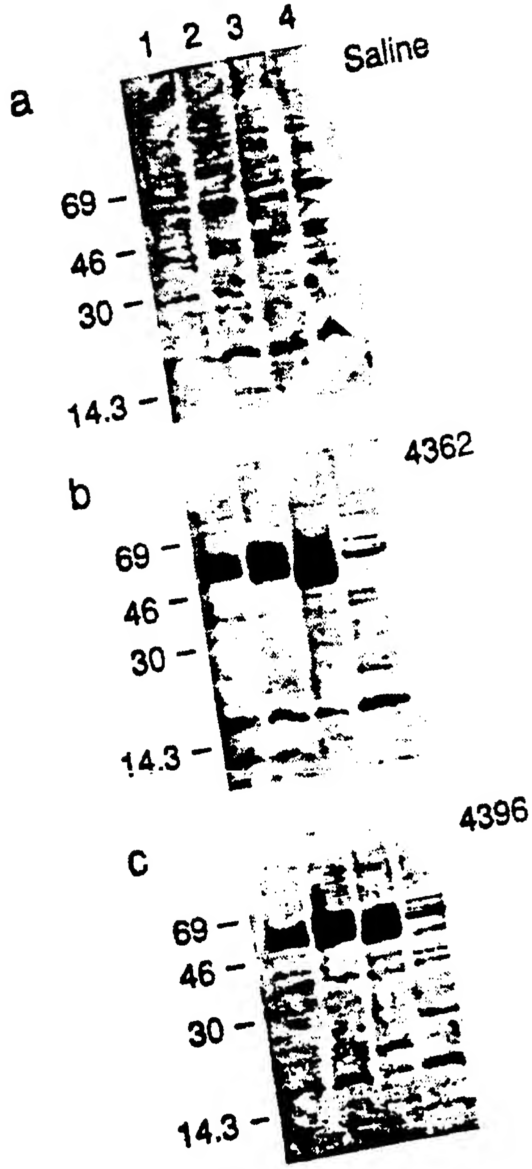


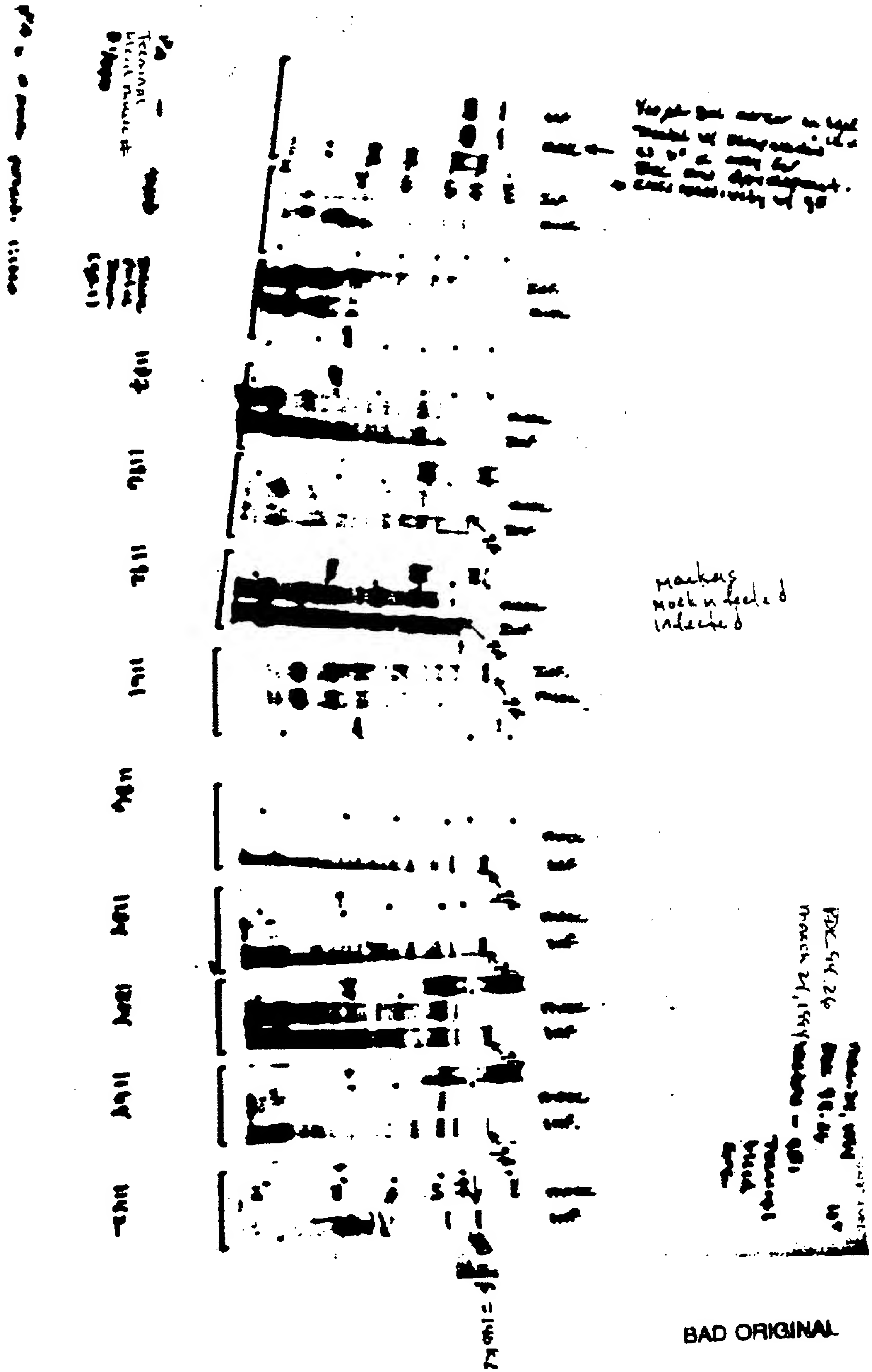
FIG. 1B



BAD ORIGINAL



BAD ORIGINAL



BAD ORIGINAL

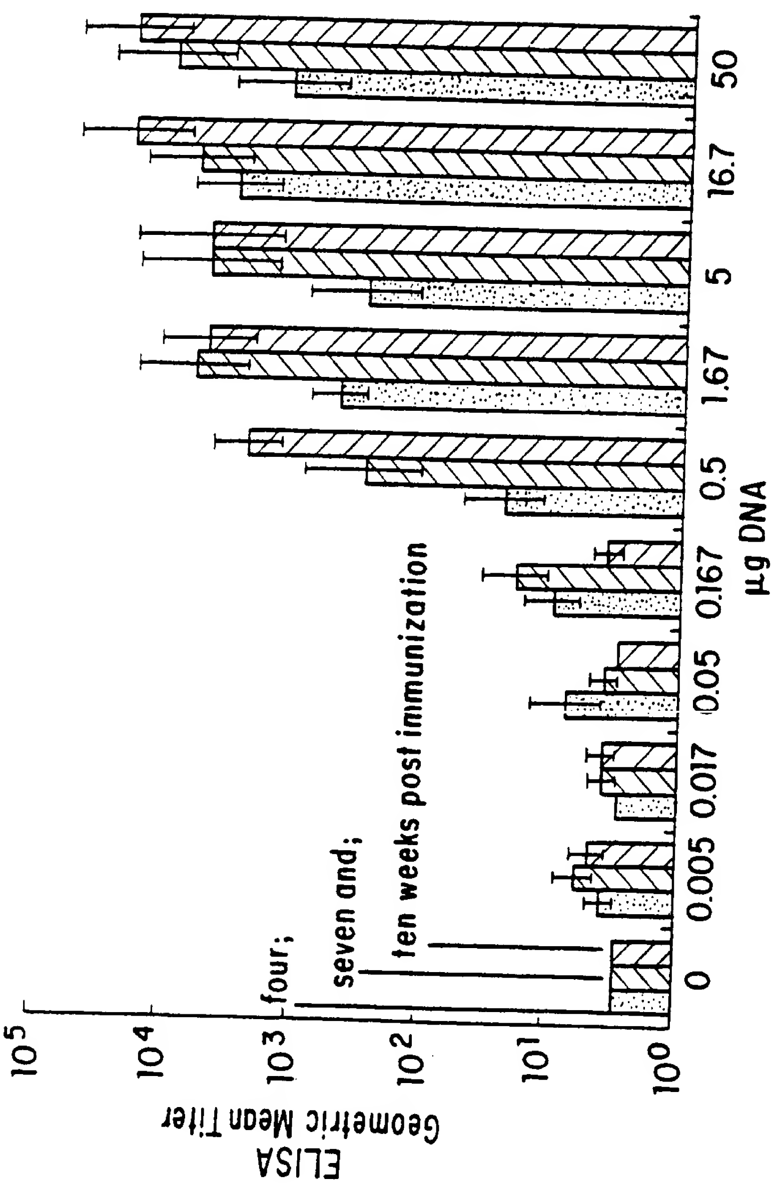

ELISA
Geometric Mean Titer
10^5
10^4
10^3
10^2
10^1
10^0
four;
seven and;
ten weeks post immunization
0
0.005
0.017
0.05
0.167
0.5
1.67
5
16.7
50
μg DNA

FIG. 3

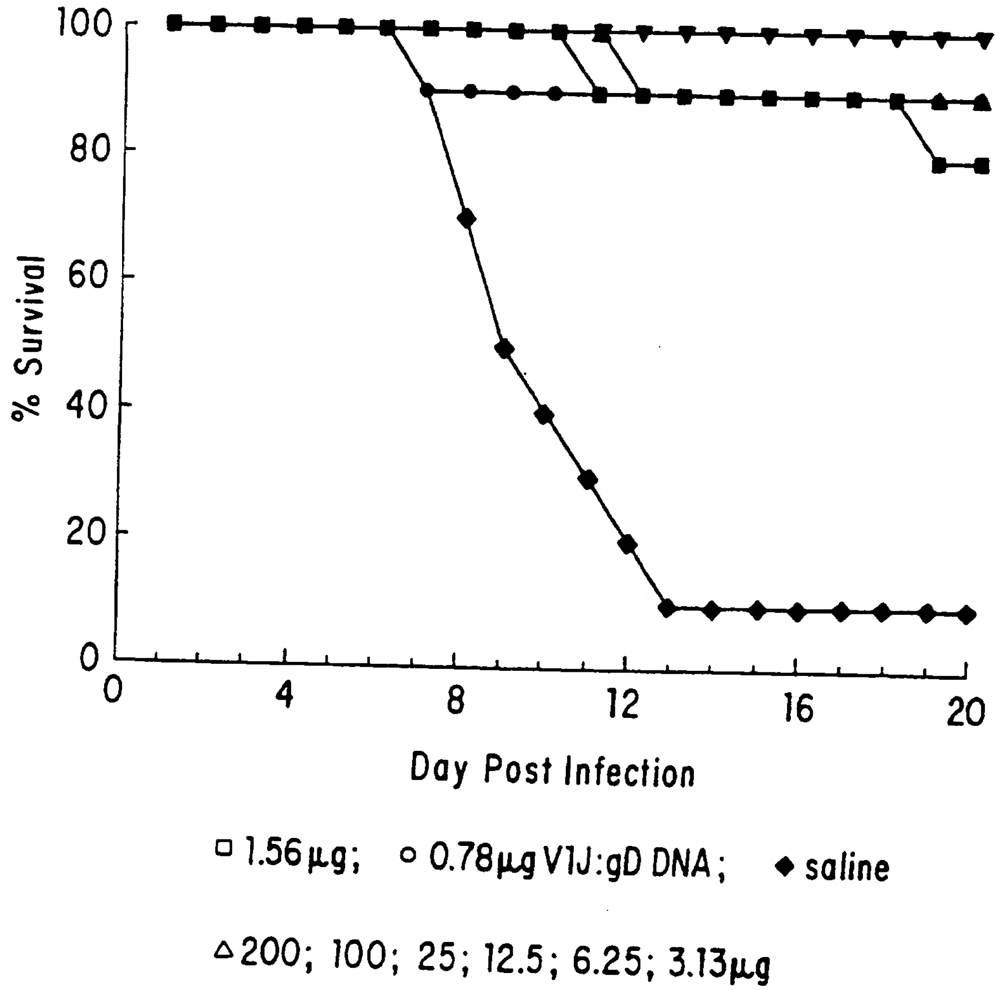

100
80
60
40
20
0
% Survival
0
4
8
12
16
20
Day Post Infection
□ 1.56μg; ○ 0.78μg V1J:gD DNA; ◆ saline
△200; 100; 25; 12.5; 6.25; 3.13μg

FIG. 4

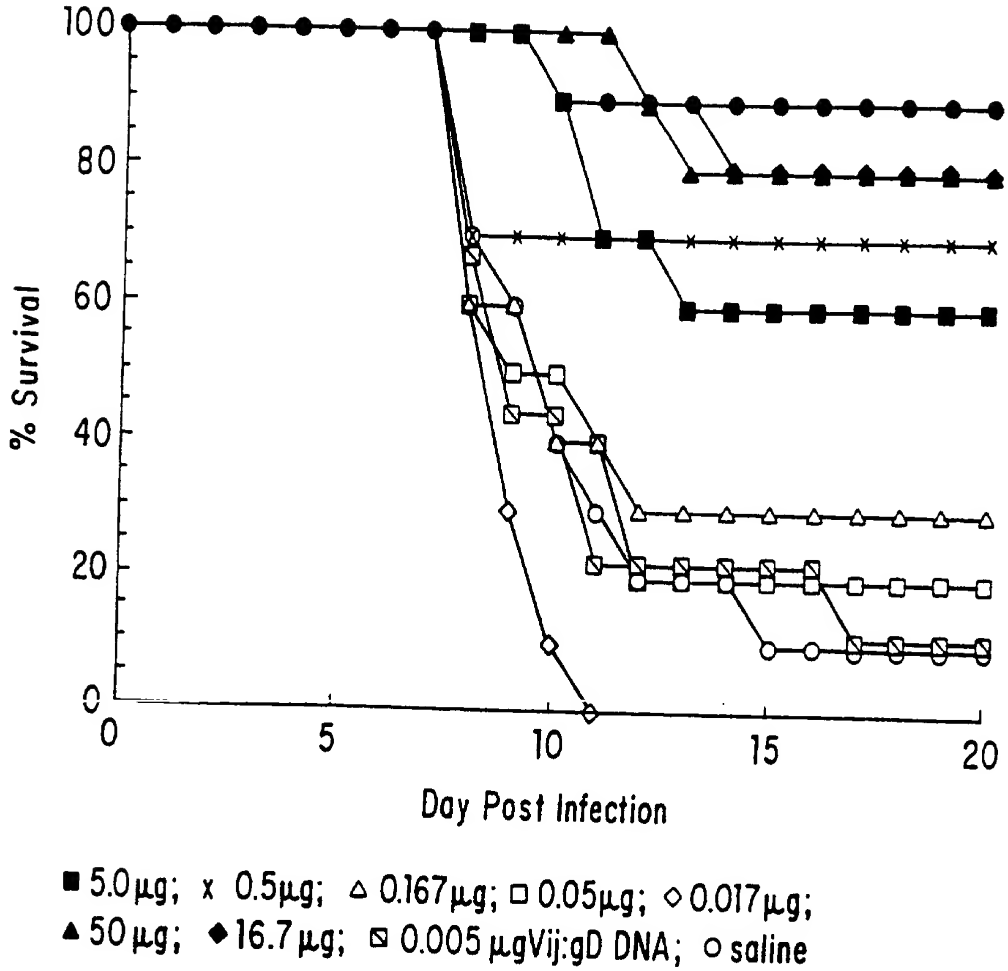

100
80
60
40
20
0
% Survival
0
5
10
15
20
Day Post Infection
■ 5.0μg; x 0.5μg; △ 0.167μg; □ 0.05μg; ◇ 0.017μg;
▲ 50μg; ◆ 16.7μg; ⊠ 0.005 μgVij:gD DNA; ○ saline

FIG. 5

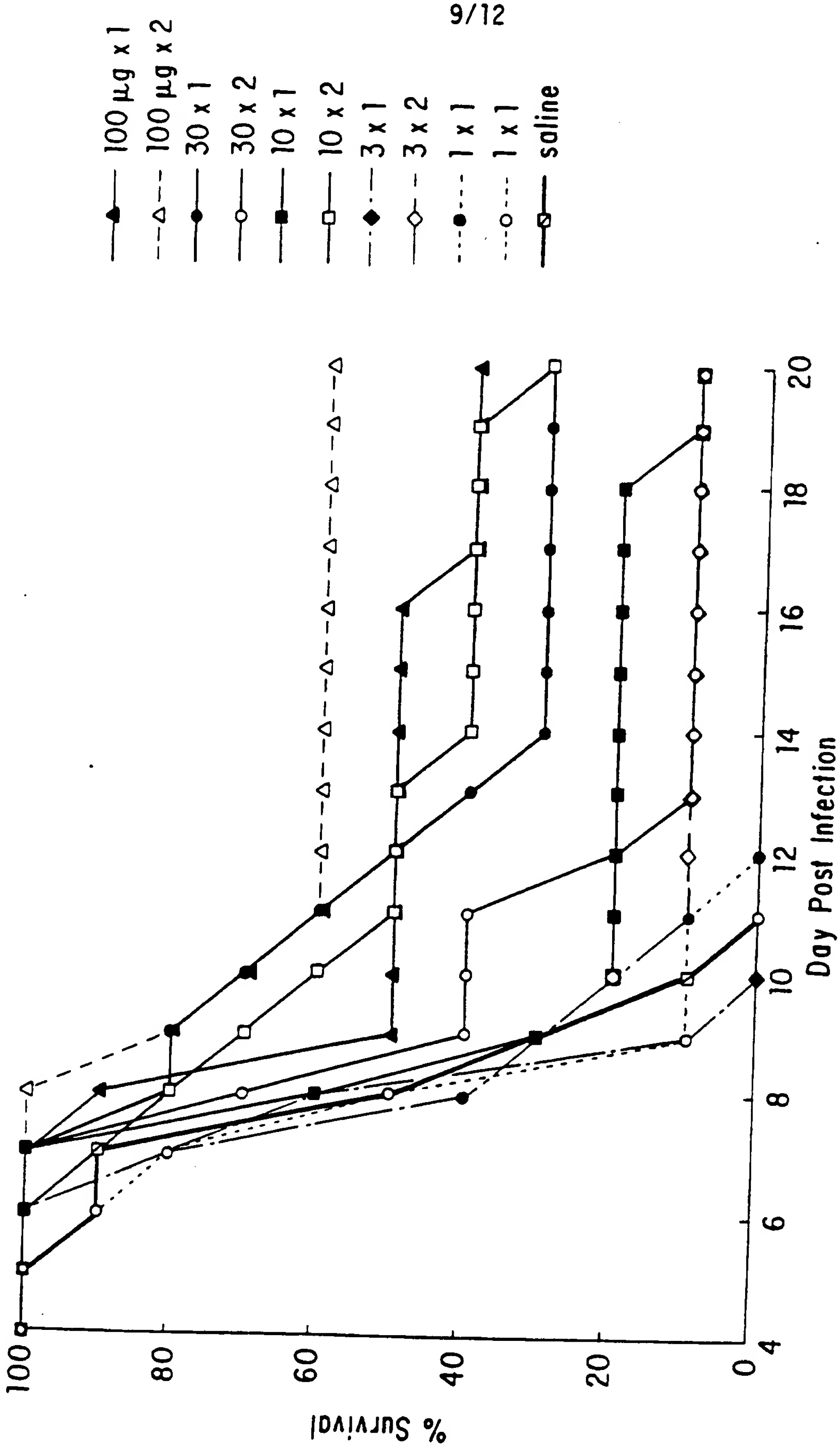

100 µg x 1
100 µg x 2
30 x 1
30 x 2
10 x 1
10 x 2
3 x 1
3 x 2
1 x 1
1 x 1
saline
% Survival
100
80
60
40
20
0
4
6
8
10
12
14
16
18
20
Day Post Infection

FIG. 6

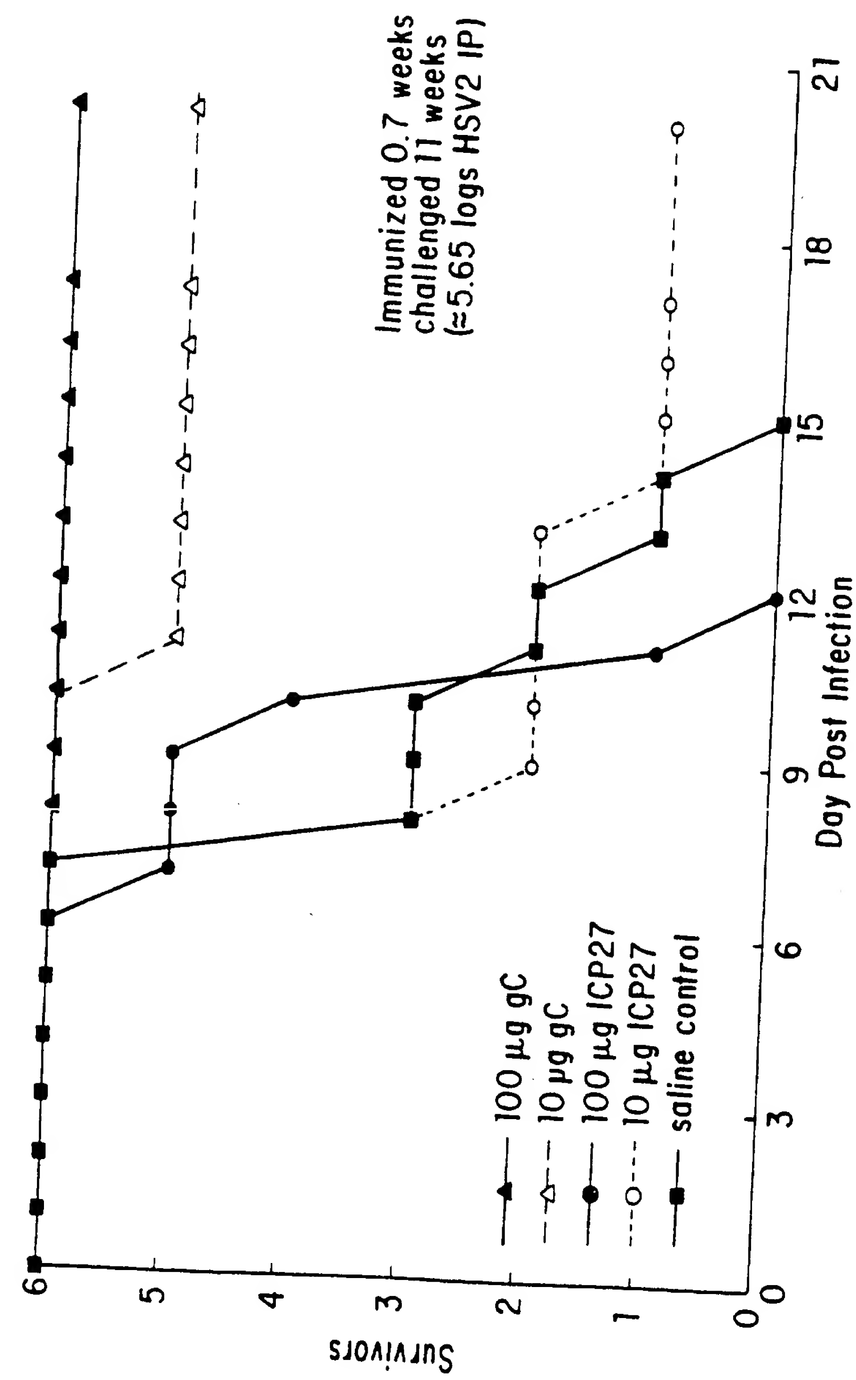

Immunized 0.7 weeks
challenged 11 weeks
(≈5.65 logs HSV2 IP)
100 μg gC
10 μg gC
100 μg ICP27
10 μg ICP27
saline control
Survivors
Day Post Infection
0
1
2
3
4
5
6
0
3
6
9
12
15
18
21

FIG. 7

Group[a]	Survivors/ Total (%)	Mean Day to Death	Paralyzed/ Total (%)	Vaginal Virus Titer[b]		
				Day 2[c]	Day 4	Day 6
Vaccine, 10 μg	8/10 (80)	12.5 ± 0.7	5/10 (50)	3.8 ± 1.9	2.3 ± 1.2	<1.5 ± 0.0
Vaccine, 100 μg	10/10 (100)†	>21	0/10 (0)*	3.0 ± 1.3Ø	2.0 ± 0.7†	<1.5 ± 0.0
Placebo	6/10 (60)	14.8 ± 4.0	8/10 (80)	5.0 ± 2.3	3.1 ± 1.4	1.6 ± 0.3

a The vaccine was administered intramuscularly 11 and 4 weeks prior to virus challenge.
b Log_{10} cell culture infections doses per ml, determined from vaginal swabs.
c After virus inoculation.
* P<0.001.
† P=008, Ø P=0.06.

FIG. 8

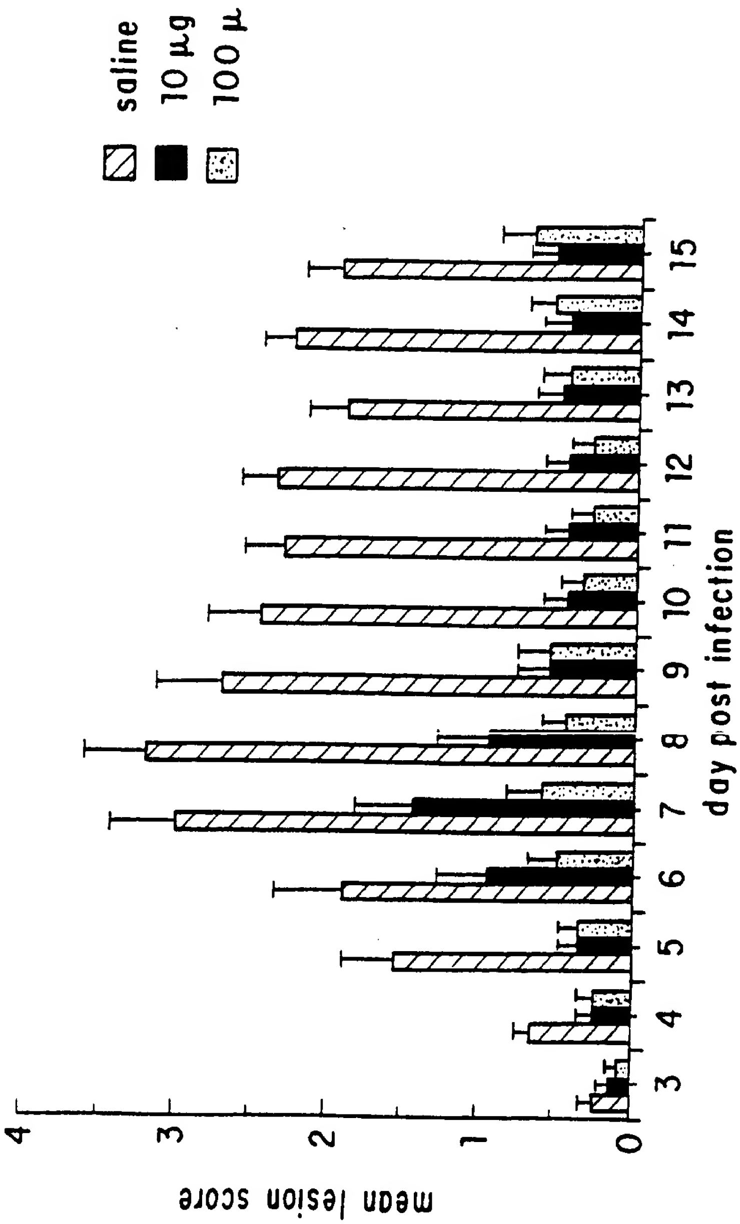

saline
10 µg
100 µ
mean lesion score
0
1
2
3
4
day post infection
3
4
5
6
7
8
9
10
11
12
13
14
15

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/09057

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9011092	04-10-90	AU-B-	5344190	22-10-90
		EP-A-	0465529	15-01-92
		JP-T-	4504125	23-07-92
WO-A-8800971	11-02-88	AU-B-	612983	25-07-91
		AU-B-	7789987	24-02-88
		EP-A-	0275300	27-07-88
		JP-T-	1500755	16-03-89
EP-A-0406857	09-01-91	AT-T-	123065	15-06-95
		CA-A-	2020668	08-01-91
		DE-D-	69019609	29-06-95
		JP-A-	4117399	17-04-92

.rnational application No.

PCT/US95/09057

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 3,5,10-11
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 3,5,10-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/compsition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	ANTIVIRAL RESEARCH, (1995) 28/2, 147-157, GHIASI, H. ET AL. 'Vaccination of mice with herpes simplex virus type 1 glycoprotein D DNA produces low levels of protection against lethal HSV -1 challenge.' see the whole document	1-11
T	JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, (1995) 273/18, 1403-1404, MARWICK, C. 'Exciting potential of DNA vaccines explored.' see the whole document	1-11
T	INT.PHARM.J.;(1995) 9, SUPPL.1, 10 , LIU, M. ET AL. 'Immune responses and pre-clinical efficacy of DNA vaccines for viral diseases' see the whole document	1,2,4,5, 10,11

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,88 00971 (COMMW SCIENT IND RES ORG ;UNIV AUSTRALIAN (AU)) 11 February 1988 see page 3, line 6 - page 4, line 25 see claims ---	6,7
A	EP,A,0 406 857 (TAKEDA CHEMICAL INDUSTRIES LTD) 9 January 1991 see page 3, line 25 - page 4, line 20 see examples 2,3,7 ---	1-11
A	JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, 271/12, 929-31, March 1994 ELLIS, R. & DOUGLAS, R. 'New vaccine technologies' see page 931, left column, last paragraph - right column ---	1-11
A	VACCINE, (1992) 10 (4) 209-13, HO, R. ET AL. 'Liposome-formulated interleukin-2 as an adjuvant of recombinant HSV glycoprotein gD for the treatment of recurrent genital HSV -2 in guinea-pigs.' see the whole document ---	6-9
A	SCIENCE, vol. 259, 16 June 1989 US, pages 1275-1281, FRIEDMANN, T. 'Progress toward human gene therapy' cited in the application see page 1276, left column, paragraph 1 see page 1279, right column, line 31 - page 1280 ---	1
A	BIOTECHNOLOGY, vol. 11, October 1993 NEW YORK US, pages 1117-1119, EDGINGTON, S. 'turning on tumor-fighting T-cells' cited in the application see the whole document ---	7
P,X	FASEB JOURNAL 9 (3). 1995. A207, MANICKAN, E. ET AL. 'Protection against HSV infection by DNA vaccination: Plasmid DNA encoding HSV -1 gB protects mice from HSV -1 zosteriform lesions.' see the whole document & EXPERIMENTAL BIOLOGY 95, PART I, ATLANTA, GEORGIA, USA, APRIL 9-13, 1995., --- -/--	1,2,5